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FAILURE OF FOLIC ACID ANTAGONIST TO INTERFERE WITH THE ACTION OF TESTOSTERONE PROPIONATE ON THE COMBS AND TESTES OF YOUNG COCKERELS[1]

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THE failure to obtain growth of the female reproductive tract after treatment with estrogen in the absence of folic acid has been well established. Since the initial report of Hertz and Sebrell (1944) on the inability of estrogen to stimulate growth of the oviduct of folic acid deficient chicks, confirmation has been obtained with the use of both synthetic diets and folic acid analogues. This inhibition of the growth promoting action of estrogen on the female reproductive tract has been observed in the frog (Goldsmith, Schreiber and Nigrelli, 1948), the chick (Hertz and Sebrell, 1944; Hertz, 1945, 1948; Andrus and Zarrow, 1949), the rat (Hertz and Tullner, 1949) and the monkey (Hertz, 1948; Zarrow, Hisaw and Salhanick, 1949).

The role of folic acid in androgen induced growth of the male accessories has not been so clearly defined. Goldsmith, Black and Nigrelli (1949) have reported a failure to obtain androgen stimulated growth of the prostate in folic acid deficient mice, while Brendler (1949) has indicated that aminopterin had no effect on the growth stimulating action of testosterone on the prostate of rats. Preliminary reports on the cockerel have indicated that in this species also, a lack of folic acid failed to inhibit androgen stimulated growth of the comb (Dorfman, 1948; Zarrow and Koretsky, 1949). Haque *et al.* (1949) reported that stimulation of the comb of chicks with testosterone propionate was not prevented by any of the known vitamin deficiencies.

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The present investigation was undertaken to examine in greater detail the role of folic acid in the growth of the comb and inhibition of the testes of young cockerels treated with testosterone propionate. A folic acid deficient state was induced in the chicks both by the use of a synthetic diet free of the vitamin and by the addition of a folic acid analogue to the normal diet.

MATERIALS AND METHODS

White leghorn cockerels of selected stock, obtained from the Hall Brothers Hatchery,[3] were used in these experiments. The birds were shipped on the day of hatching and treatment was started on the second day of age. The chicks were kept in cages with a wire mesh bottom and uniform lighting and heating. Subcutaneous injections of testosterone propionate were given daily in the thigh region of either leg, alternating the site of injection. The hormone was dissolved in sesame oil and the birds were injected with 0.05 ml. of oil daily, containing 100 μg. of testosterone propionate.

The cockerels were fed a normal starter ration to which was added the folic acid antagonist[4] (crude methyl folic acid) as a 2 or 4 per cent mixture. All cockerels were given food *ad libidum* except for those on a restricted diet in which instance the amount of food was limited so as to obtain comparable body weights between the controls and the folic acid deficient birds. A synthetic diet was also used to produce a folic acid deficiency. The composition of this diet (Campbell, Brown and Emmett, 1944) was as follows: purified casein, 25%; gelatin, 10%; L-cystine, 0.3%, choline chloride, 0.2%; corn starch 52.4%; cellulose, 3.0% Crisco, 4.0%; manganese sulfate, 0.1%; and salts 5%. All known vitamins except folic acid were added in the following amounts per 100 grams of diet; 1600 U.S.P. units of vitamin A, 160 U.S.P. units of vitamin D, 24 mgm. of vitamin E, 5 μg. of Vitamin K, 0.4 mgm. of thiamine hydrochloride, 0.8 mgm. of riboflavin, 0.6 mgm. of pyridoxine hydrochloride, 50 mgm. of i-inositol, 15 mgm. of p-aminobenzoic acid, 2 mgm. of nicotinic acid and 1.1 mgm. of calcium pantothenate. Three μg. of crystalline biotin were given orally by dropper every second day except for one group of chicks that received 6 μg. of biotin every second day.

At autopsy, the combs and testes were removed and quickly weighed on a torsion balance. The combs were then dried for 48 hours at 110°C. and re-weighed in order to obtain the water content. Results on the weights of the combs and testes are expressed both as direct weight and as a function of body weight in order to compensate for differences in general body growth.

RESULTS

Treatment With Folic Acid Antagonist or Folic Acid Deficient Diet for 7 Days.

It is apparent from the data in Table I, that a 2% folic acid antagonist diet or a synthetic diet free of folic acid failed to interfere with the action of testosterone propionate on the comb and testes. In this experiment the

[3] Wallingford, Connecticut.

[4] The folic acid antagonist (a crude methyl folic acid preparation) was obtained through the courtesy of Dr. E. L. R. Stokstad, Lederle Laboratories, New York.

cockerels received the synthetic diet or the folic acid antagonist diet and 100 μg. of testosterone propionate for 7 days. The androgen treated chicks on either a synthetic diet free of folic acid or a diet containing folic acid antagonist had comb weights of 86.1 ± 15.4 and 54.5 ± 6.0 mgms., respectively, and the chicks on a normal or restricted diet had comb weights of 76.4 ± 8.3 and 66.5 ± 7.6 mgms. showing no significant difference between the normal and the folic acid deficient cockerels. The testes weights of the

TABLE 1. COMB GROWTH IN THE COCKEREL AFTER 7 DAYS TREATMENT WITH TESTOSTERONE PROPIONATE AND FOLIC ACID ANTAGONIST OR SYNTHETIC DIET

Diet	Treatment	No. of Chicks	Body Wt. gm.	Testis		Comb		
				Wt. mgm.	mgm. % body wt.	Wt. mgm.	mgm. % body wt.	% H_2O
Normal	—	10	78	20 *±1.62	27.8 ±1.72	21.3 ±3.6	29. ±4.0	71.4 ±1.38
2% FAA**	—	10	72	20 ±1.83	28.2 ±2.71	16.4 ±2.61	23 ±3.12	68.0 ±1.86
Synthetic	—	10	60	24 ±1.18	40. ±1.73	27. ±2.18	45. ±3.64	74.6 ±0.61
Normal	Testosterone p.	10	72	15.3 ±1.17	19.8 ±1.63	76.4 ±8.3	95.2 ±10.3	81.8 ±0.67
Restricted	Testosterone p.	10	62	11.8 ±0.96	18.9 ±1.3	66.5 ±7.6	105. ±10.	82. ±0.86
2% FAA	Testosterone p.	10	66	10.2 ±0.70	15.6 ±0.74	54.5 ±6.03	81.6 ±6.3	81.5 ±0.57
Synthetic	Testosterone p.	10	64	16. ±0.95	25.4 ±1.37	86.1 ±15.4	132.4 ±21.6	82.4 ±0.53

* Standard error $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

** Crude methyl folic acid.

androgen treated birds on folic acid antagonist were 10.2 ± 0.7 mgm. while normal, control chicks and chicks on restricted diet had testes weights of 15.3 ± 1.1 and 11.8 ± 0.9, respectively. Compared to the birds on a restricted diet, no enhancement of the depression of the testes of the folic acid deficient cockerels was found. Expression of the testes and comb weights as a function of body weight gave results comparable with the absolute weight. The combs of the chicks on a synthetic diet and treated with testosterone propionate were 25 per cent heavier than the combs of restricted controls. However, this increase was not statistically significant due to the high degree of variation in comb weights. Increase in water

content of the comb occurred in all chicks receiving androgen regardless of the folic acid deficiency.

Treatment of young cockerels for 7 days with 100 μg. of testosterone propionate and a diet containing 4% methyl folic acid again failed to prevent comb growth and depression of testes weights (Table 2). The combs of the birds on a normal diet or a restricted diet and treated with testosterone propionate weighed 83.1 and 66.5 mgm. respectively whereas the combs of the folic acid deficient chicks treated with an equal amount of androgen weighed 109.2 mgm. Comparison of the comb weights as a function of body weight for the three groups of cockerels increased the disparity between the chicks on the normal diet and the folic acid antagonist

TABLE 2. ACTION OF TESTOSTERONE PROPIONATE ON COMBS OF COCKERELS FED A 4% FOLIC ACID ANTAGONIST DIET FOR 7 DAYS

Diet	Treatment	No. of Chicks	Body Wt. gm.	Testis		Comb		
				Wt. mgm.	mgm. % body wt.	Wt. mgm.	mgm. % body wt.	% H_2O
Normal	—	18	84	29.5	35.1	34.	40	75
Normal	Testosterone p.	5	92	21.6	23.4	83.1	90	82.9
Restricted	Testosterone p.	10	62	11.8	18.9	66.5	105	82.4
4% FAA	—	9	66	18.5	28.	24.	37	76.9
4% FAA	Testosterone p.	9	65	10.5	16.1	109.2	168	83.2

diet but did not affect the difference between the chicks on the restricted diet and the folic acid antagonist diet. The androgen treated cockerels on a folic acid deficient diet possessed comb weights of 168 mgm. per cent body weight, those on a normal diet had comb weights of 90 mgm. per cent and those on a restricted diet had comb weights of 105 mgm. per cent. Thus the combs of the androgen treated, folic acid deficient birds were 60 per cent heavier than the combs of the androgen treated cockerels on a restricted diet when compared either as an absolute weight or as a percentage of the body weight. However due to the disparity in body weight between the normal controls and the folic acid deficient birds, the difference in comb size is increased when compared as a function of body weight. Although in an absolute sense the combs of the androgen treated deficient birds are only 27 per cent heavier than the normal androgen controls, if the body weight is considered the percentage difference increases to 81 per cent.

Again a depression in testis weight was observed after treatment with testosterone propionate. The amount of inhibition was the same in both the restricted diet and folic acid deficient cockerels. No significant changes

were noted in the water content of the comb. Testosterone propionate caused an increase in water content of both the normal and the folic acid deficient chick.

Folic Acid Deficient Diet for 14 days

Due to the high degree of toxicity of the 4% methyl folic acid diet, a third experiment was carried out in which the cockerels were fed a synthetic diet free of folic acid for 14 days (Table 3). Again the birds were

TABLE 3. COMB GROWTH IN THE FOLIC ACID DEFICIENT COCKEREL AFTER TREATMENT WITH TESTOSTERONE PROPIONATE FOR 14 DAYS

Diet	Treatment	No. of Chicks	Body Wt. gm.	Testis		Comb		
				Wt. mgm.	mgm. % body wt.	Wt. mgm.	mgm. % body wt.	% H_2O
Restricted	—	10	77.6	15.5 ±0.86	20.0 ±0.8	47.4 ±7.45	59.7 ±8.75	73.0 ±0.40
Synthetic	—	8	69.8	28. ±3.6	39.7 ±4.0	31.6 ±3.8	43.1 ±4.7	76.9 ±0.54
Restricted	Testosterone p.	9	76.9	12.0 ±0.58	16.2 ±1.34	595.0 ±50.8	779. ±66.3	88.3 ±0.35
Normal	Testosterone p.	10	129.	22.1 ±2.73	17.0 ±1.71	1056. ±90.9	811. ±49	86.2 ±0.47
Synthetic* (A)	Testosterone p.	6	60	13.5 ±1.22	22.5 ±1.71	347.9 ±22.0	588. ±51.8	88.2 ±1.4
Synthetic** (B)	Testosterone p.	10	103.	19.1 ±1.7	18.8 ±1.63	1456 ±131	1372. ±168	90.0 ±0.42

* Received 3 μg. of biotin every second day.
** Received 6 μg. of biotin every second day.

injected daily with 100 μg. of testosterone propionate for 14 days and autopsied on the 15th day. Cockerels on a normal or restricted diet and receiving androgen possessed combs weighing 1056±90 and 595±50 mgms. respectively, or 779±66 and 811±49 mgm. per cent body weight in grams. It is apparent that no significant difference exists between the two groups when the values are expressed as a function of body weight in order to compensate for the decreased growth of the group on a restricted diet.

Two groups of chicks on the synthetic diet free of folic acid were injected with 100 μg. of testosterone propionate daily. Group A received 3 μg. of biotin every second day and possessed comb weights of 347.9±22 mgms., whereas group B received 6 μg. of biotin every second day and showed comb weights of 1456±131 mgm. In spite of the fact that group

B had an average body weight of 103 grams at autopsy and group A 60 grams, comparison of the comb weights as a per cent of body weight indicates that the combs of group B (1372 mgm. %) were twice as large as the combs of group A (588 mgm. %). The data further indicate that the combs of group B are significantly larger than the combs of the androgen treated controls. The testes were inhibited in all groups treated with testosterone propionate. Likewise an increased water content is observed in the combs of all androgen treated groups although the greatest amount is seen in the group treated with androgen and 6 μg. of biotin. Comparison of group B with the androgen treated controls shows the former to have significantly larger combs. Testes weights and water content of the combs showed no significant changes with regard to folic acid deficiency.

DISCUSSION

The adequacy of both the folic acid antagonist and the folic acid free diet to produce a deficiency of this vitamin was apparent from the failure of the birds to show normal body growth and to maintain normal hemoglobin values. A 20 to 35 per cent decrease in the hemoglobin concentration was observed at autopsy in the folic acid deficient cockerels when compared with the normal controls. In several instances attempts to maintain the cockerels on the 2% antagonist diet for 3 weeks or the 4% antagonist diet for 2 weeks resulted in a high mortality.

It is apparent from an examination of these data that folic acid is not essential for androgen stimulated, comb growth in the cockerel. Contrary to the results obtained on the estrogen stimulated reproductive tract of the female (Hertz, 1948a) and on the action of androgen on the prostate of the folic acid deficient mouse (Goldsmith, Black & Nigerilli, 1949), androgen produced unquestionable comb growth in the vitamin deficient cockerel. These results are in agreement with the report of Dorfman (1948), Zarrow and Koretsky (1949), and Haque *et al.* (1949), on cockerels and Brendler (1949), on rats and indicate that a sex difference exists with regard to the relationship of folic acid to the growth promoting action of estrogens and androgens. It would appear that while folic acid is essential for the growth stimulating action of estrogen on the oviduct, the vitamin is not essential for the growth stimulating action of androgen on the comb and accessory tissues. The failure of the folic acid deficient mouse to respond to androgen is difficult to explain in view of the responses noted in both the rat and the cockerel. It has been pointed out recently, however, that in extreme inanition the accessory sex organs of the mouse failed to respond normally to testosterone propionate (Goldsmith & Nigrelli 1950). This might indicate the need for distinguishing between vitamin deficiency and inanition.

That a sex difference exists with regard to the action of folic acid is also noted in the ability of male mice to tolerate dosages of pteroylglutamic acid that are lethal to female mice (Talyor and Carmichael, 1949) and in the increased susceptibility of the cockerel to methyl folic acid as compared with the female chick (Zarrow, 1950).

An enhancement of the action of testosterone propionate on the comb of the cockerel was noted in two of the three experiments. In one instance the increased response was obtained with a 4 per cent folic antagonist diet. It is conceivable that the failure to obtain the same results with a 2% antagonist diet is simply due to the degree of deficiency produced by the diet. The present data do not lend themselves to an explanation of this enhanced response but it would appear to be due either to an interference in the degradation of the androgen or an increased sensitivity of the comb. Breneman (1942) reported an increased comb response to androgen in the starved cockerel. That this was not observed in the chicks on the restricted diet, is probably due to the fact that the degree of inanition was not as great in these experiments and would indicate that the enhanced response is not an inanition effect. It is rather significant that in the 14 day experiment the enhanced action of androgen is seen only when an increased supplement of biotin is given the cockerels. This is suggestive of a fundamental need of biotin in order to obtain maximum growth of the comb although Haque *et al.* (1949) reported comb growth in the absence of biotin.

The weight of the testes was depressed by the androgen in both the control and folic acid deficient cockerels. No uniformity was noted with regard to the degree of atrophy, i.e. the cockerels with the largest combs did not have the smallest testes (Table 3).

SUMMARY

Androgen treatment produced unquestionable comb growth and inhibition of the testes in cockerels placed on folic acid deficient diet or a diet containing a folic acid analogue (methyl folic acid). In a deficiency induced by a 4% methyl folic acid diet or a folic acid free diet for 14 days and supplemented by high doses of biotin, the effect of androgens on the comb was enhanced.

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THE INHIBITION OF GONADOTROPHIC HORMONE SECRETION BY PHYSIOLOGICAL DOSES OF ESTROGEN*

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WHEN an immature female rat is united in parabiosis with an ovariectomized littermate, the ovaries of the intact rat undergo hypertrophy (Kallas, 1930). This has been attributed to the hypersecretion of gonadotrophins by the pituitary of the castrated rat, the hormones of which pass directly via the blood to the intact partner where they stimulate the ovaries (Bunster and Meyer, 1938; Biddulph, Meyer and Gumbreck, 1940). It is believed that the gonadotrophin which increases in the ovariectomized female is largely follicle stimulating hormone (Greep, 1940; Biddulph and Meyer, 1946). Daily injections of small quantities of estrogen, or larger quantities of androgen, desoxycorticosterone acetate, or progresterone into the ovariectomized partner prevent the hypersecretion of the gonadotrophic hormones by the pituitary and the corresponding hypertrophy of the ovaries of the partner (Bunster and Meyer, 1933; Biddulph, Meyer and Gumbreck, 1940; Byrnes and Shipley, 1950). This phenomenon provides a means whereby one can assay the gonadotrophins being secreted by the pituitary of the ovariectomized female, and also the extent to which this secretion is inhibited by the administration of estrogen. The uterus of the castrate partner can be used to measure the uterine stimulating activity of the estrogen simultaneously with its power to inhibit gonadotrophic hormone secretion.

The purpose of this paper is to describe results of experiments designed to study the relationship between the amount of estrogen required to inhibit gonadotrophic hormone secretion and the amount of estrogen required to stimulate the uterus in immature female rats.

MATERIALS AND METHODS

Thirty-day old female littermate rats of the Sprague-Dawley strain weighing between 65 and 75 gm. were joined in parabiosis according to the methods of Bunster and Meyer (1933), except that metal skin clips were used instead of silk sutures in closing skin

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incisions. The right partners were ovariectomized at the time of the parabiotic union. Twenty-three pairs were left uninjected to serve as controls; the remainder were used to test two natural estrogens, alpha-estradiol and estrone, and one synthetic estrogen, 3-(p-hydroxyphenyl)-4-(p-acetylphenyl) hexane hereafter referred to as compound #15.[1] The hormone being tested was dissolved in corn oil and 0.1 cc. of the oil was injected subcutaneously into the ovariectomized right partner daily for 10 days, beginning with the day of operation. On the 11th day the rats were killed, and the ovarian and uterine weights were determined. Although different amounts of hormone were injected, the quantity of the oil was kept constant. The corn oil has been assayed and found to be physiologically inactive in these procedures.

TABLE 1. THE UTERINE AND OVARIAN WEIGHTS OBTAINED IN PARABIOTIC RATS GIVEN ESTROGEN

Compound	Dose (μg/day)	No. Prs.	Average organ weights	
			Ovaries[1]	Uterus[2]
Uninjected	—	23	160 mg. (110–230)	52 mg. (32–90)
Alpha-estradiol	.0032	2	146	47
	.0065	2	122	60
	.0065	1	15	43
	.009	2	16	45
	.012	3	25	51
	.020	3	20	65
	.025	3	25	135
	.050	2	29	168
Estrone	.050	3	112	51
	.075	2	30	48
	.10	6	34	52
	.15	3	40	56
	.20	5	42	131
	1.00	3	31	217
Compound #15	5.00	2	134	75
	25.00	3	152	70
	50.00	2	22	61
	100.	3	24	65
	200.	2	30	85
	250.	2	32	138
	500.	2	29	148

[1] Ovaries of the left, intact partner.
[2] Uterus of the right, ovariectomized, injected partner.

RESULTS AND DISCUSSION

It is to be emphasized that the average weight of the hypertrophied ovaries of the lefthand partner was 160 mg. (Table 1), whereas with parabionts used in earlier reports from this laboratory (Biddulph, Meyer and Gumbreck, 1940), the average ovarian weight was 85 mg. Also, in the experiments being reported here, the pituitary secretion of gonadotrophic

[1] We are indebted to Dr. A. L. Wilds of the University of Wisconsin Chemistry Department for supplying this compound.

hormone was inhibited with 0.009 μg. per day of alpha-estradiol, while formerly 0.025 μg. per day was necessary.

In earlier experiments (Meyer and Biddulph, 1941), it was found that alpha-estradiol injected into one partner of castrated female parabiotic rats at a dose of 1.0 μg. per day for 10 days caused uterine growth in the opposite partner. In repeating this work, it was found that when the estrogen was injected into the right partner at 0.5 μg. per day there was slight stimulation of the uterus of the left partner. When 0.7 μg. per day was injected, there was moderate uterine stimulation.

Both groups of rats were obtained directly from Sprague-Dawley, Inc., but since the first experiments were done, a new colony of Sprague-Dawley rats has been established from caesarean sections and has been maintained parasite free. In addition, it is probable that some selection has been practiced and that the present stock is not physiologically the same as it was. On the basis of our data, it appears that the gonadotrophic activity of the present strain is greater and that the pituitary is more sensitive to the inhibitory action of estrogens. We are forced to this conclusion since the methods and other variables in the experiment have not been changed to the best of our knowledge.

When 30-day female rats were united in parabiosis and neither partner ovariectomized, the ovarian weights of five pairs averaged 17 mg. when the rats were autopsied after 10 days. This was not only less than 41 mg. which was the average ovarian weight of normal rats of this age, but less than 26 mg. which was the average weight of the ovaries of 30-day old females. It is seen that the ovaries not only failed to grow in the ten day period, but atrophied. The failure of the ovaries of the parabiotic rats to grow at a normal rate may be due to a decreased gonadotrophic hormone secretion as part of the adaptation syndrome initiated by the operative procedures (Selye, 1946). It follows from this that in females in parabiosis with an ovariectomized littermate, ovarian weights of more than 17 mg. are evidence of increased gonadotrophic hormone from the partner.

In the experiments being reported in this paper, ovarian weights of between 50 and 100 mg. are considered to indicate partial inhibition of pituitary gonadotrophic hormone secretion, and ovarian weights of less than 50 mg. as indicative that pituitary inhibition was almost complete. All the compounds reported here had strong inhibitory effects upon the pituitary so that ovarian weights representing partial pituitary inhibition were not found. In as much as uterine weights of 90 mg. were obtained in the uninjected controls, uterine weights of more than 100 mg. are considered evidence of definite uterine stimulation.

As is shown in Table 1, when the estrogen was injected at low doses, there was a range in which inhibition of the pituitary gonadotrophic hor-

mone occurred without uterine stimulation. Alpha-estradiol and estrone inhibited the secretion at approximately one-third the uterine stimulating dose, and compound #15 inhibited at about one-fourth the dose that produced uterine growth.

These data provide the basis for the generalization that the pituitary secretion of gonadotrophic hormone is inhibited by estrogen in *immature female rats* at smaller doses than which stimulate the uterus.

SUMMARY

When natural and synthetic estrogens were injected into immature ovariectomized rats in parabiosis with intact females, the gonadotrophic hormone secretion was inhibited by the hormones at levels below those causing uterine stimulation. It is suggested that in intact *immature* female rats, gonadotrophic secretion is inhibited by estrogen at very low and physiological levels.

Although the trauma inherent in the parabiosis procedure was found to cause some ovarian atrophy, this was more than compensated for by the hypersecretion of gonadotrophin when one of the rats was ovariectomized.

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A METHOD FOR MEASURING AND THE EFFECT OF ADRENOCORTICOTROPIC HORMONE ON LIPID-SOLUBLE REDUCING SUBSTANCES OF RABBIT PLASMA*

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SATISFACTORY results have been attained in measuring cortin-like substances in urine by chemical methods. It has been possible to develop one of these based on the reduction of phosphomolybdic acid (Heard and Sobel, 1946), so that we can measure the concentration of lipid-soluble reducing substances in blood samples of reasonable size. With this method it is possible to recover 11-desoxy-corticosterone, corticosterone, 17-hydroxy-11-dehydrocorticosterone (Compound E), and the reducing compounds in cortical extracts from bovine adrenal glands, when added to rabbit blood plasma.

Measured with a chemical method the excretion of cortin-like substances in the urine of human subjects is markedly increased by the administration of anterior pituitary adrenocorticotropic hormone (ACTH) (Mason *et al.*, 1948; Forsham *et al.*, 1948; Romanoff *et al.*, 1949; Sprague *et al.*, 1950). With our method we have found similar increases in blood plasma values using the rabbit as the experimental animal.

EXPERIMENTAL

Reagents

Petroleum ether (40–70°C.) is redistilled.

Ethyl alcohol (absolute) is redistilled.

Acetone redistilled from K MnO_4.

Chloroform is washed with $NaHCO_3$ and H_2O and redistilled. This solvent is prepared each week and stored in amber bottles.

Glacial acetic acid is redistilled from chromic acid.

Phosphomolybdic acid solution is prepared according to Folin and Wu (1920) without the sodium tungstate.

Phosphomolybdic acid reagent is prepared with equal volumes of glacial acetic and phosphomolybdic acid solution.

Extraction of Plasma. Five ml. of plasma which is obtained from heparinized blood is

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used for each determination. In all cases duplicate determinations are carried out. The proteins are removed by the addition of 3 volumes of absolute ethyl alcohol. The solution is centrifuged and the supernatant is transferred to an 80 ml. centrifuge tube. The precipitate is re-extracted 2 times with 10 ml. aliquots of 75 per cent alcohol. Before the centrifugation of each wash the precipitate is disintegrated and thoroughly mixed with a stirring rod. The combined 75 per cent alcohol extracts are extracted five times with 20 ml. aliquots of petroleum ether. The petroleum ether is drawn off each time with a suction pipette and discarded. The alcohol extract is then transferred with the aid of 15 ml. of water to a liter flask of an all-glass distilling apparatus. The solution is concentrated to 3–5 ml. under reduced pressure in a stream of nitrogen at a bath temperature below 40°C. The receiver is immersed in crushed dry ice. The residue is transferred to a separatory funnel with a pipette using sufficient wash water so that the final volume is approximately 50 ml. The water solution is extracted three times with 50 ml. aliquots of chloroform and each time the chloroform is drained into a 220 ml. centrifuge bottle. The bottle containing the chloroform extracts is cooled in a water-ice bath and washed with 15 ml. of chilled 0.1 N NaOH in 25 per cent NaCl and five times with 15 ml. aliquots of an aqueous solution of 25 per cent NaCl. It is necessary to centrifuge each time although the NaCl aids in breaking the emulsions. The centrifuge tube is kept in the ice bath between washings. The alkaline and salt water washes are drawn off with a suction pipette and discarded. The chloroform solution is dried with 1.5 grams of anhydrous $MgSO_4$. After drying for three hours or longer the $MgSO_4$ is removed by filtration through a fritted disc pressure filter and washed with a small quantity of dried $CHCl_3$. The chloroform solution, which is filtered directly into a liter distilling flask, is concentrated to dryness under nitrogen and vacuum as carried out for the alcohol concentration. The residue is leached with five 3-ml. portions of acetone and transferred each time with a pipette to a 6 ml. capacity micro-reaction beaker having a fritted glass filtering attachment. Slight pressure is applied with a rubber bulb and the acetone is filtered into a test tube. The test tube is placed in a 25°C. water bath and the acetone removed in a stream of nitrogen.

Method of Color Development. The reducing values are determined by the method of Heard and Sobel (1946). Six ml. of phosphomolybdic acid reagent is added to the test tube containing the plasma extract. An increase in sensitivity of about 30% was obtained by using 6 ml. of color reagent in place of the 10 ml. used in the Heard and Sobel method. A stirring rod with a flattened end is placed in each tube for mixing purposes. The tube is heated 60 minutes at 100°C. in a water bath. The water should be boiling vigorously and the solution is stirred at intervals in order to obtain maximum color development. After heating, the tube is quickly transferred to an ice bath for two minutes. The per cent transmission is then determined in a Beckman Model DU quartz spectrophotometer at 680 mu. A standard curve is made for each set of determinations by concentrating appropriate aliquots of an alcohol solution containing a standard. In these experiments 17-hydroxy-11-dehydrocorticosterone (Compound E) was used as the reference compound. Aliquots of between 10–100 gammas are sufficient.

Method for recovery of Cortin Compounds. The recoveries were determined in the following manner. Aliquots of a rabbit plasma sample were extracted and analyzed for reducing power. Simultaneously a known amount of a crystalline cortin compound was added to aliquots of the same plasma, then extracted and the reducing power measured. The steroid recovered from plasma is represented by the difference between the reducing power of the plasma before and after the steroid is added. The corticosteroids in these experiments were added in the form of 10% ethanol solutions.

RESULTS

Recovery of Added Compounds. The data given in Table 1 show that the described procedure is suitable for determining the cortin-like compounds in rabbit plasma samples. Crystalline compounds of the "salt-active" type or the 11- and 11-17-oxysteroids are recovered to the extent of 55–83 per cent. Each stage of the procedure was checked for recovery and it was found that the losses occurred largely in the NaOH and water washes of the $CHCl_3$ extract. The recovery can be improved with the usual back washing procedures, however this step was eliminated as a time-saving feature since the recovery for each pure steroid is consistent. There is no inter-

TABLE 1. THE RECOVERY OF STEROID COMPOUNDS WHEN ADDED TO RABBIT PLASMA

Steroid used	No. of det'ns	Steroid added to plasma mgs.	Steroid recovered from plasma containing added steroid mgs.	Steroid obtained from untreated plasma mgs.	Steroid[1] recovered from plasma %
11-desoxycorticosterone (DOC)	7	0.1	0.082	0.007	75
Corticosterone	7	0.1	0.107	0.024	83
17-OH-11-dehydrocorticosterone (Cpd. E)	7	0.1	0.069	0.014	55
Adrenal cortical extract 0.25 cc. extract (Upjohn) equiv. to 0.156 mg. Cpd. E	3	0.156	0.091	0.028	40

[1] The per cent recoveries for DOC were 68, 72, 75, 75, 75, 78, 82; for Corticosterone 75, 77, 78, 80, 85, 88, 91 and for Cpd. E, 47, 50, 52, 54, 57, 60, 65. The extract contains reducing substances other than corticosteroids.

ference by the plasma constituents as the recoveries from plasma or water are equally good.

Effect of ACTH Administration on Plasma Lipid-Soluble Reducing Substances. The administration of ACTH (Armour) to rabbits, as expected, increased the level of circulating plasma reducing lipids (Table 2). A substantial elevation was obtained at a dosage level of 0.4 mg/kilo and greater, whereas the administration of 0.2 mg/kilo was ineffective. In regard to the time for maximum increase after administration of ACTH, the two-hour samples gave higher plasma values than the four-hour samples. Unfortunately two-hour specimens were not taken in all experiments. In experiments 7 and 8 plasma samples were obtained 24 hours after administration of the ACTH. In each instance the level of reducing lipids had returned to normal. It is of interest that the venous plasma used in experiment 6 gave a higher value than the samples obtained simultaneously by heart puncture. Since this animal had been prepared for evisceration by the

constriction of the vena cava and had fully developed abdominal veins from which the venous blood was obtained, these results can be explained by the fact that the adrenal effluent is found in these veins. Paschkis *et al.* (1949) using a bio-assay method has reported an increase in adrenal corticoid concentration in blood from the adrenal vein after ACTH administration in the dog. Hemphill and Reiss (1947) obtained the same effect in mixed venous and arterial blood of decapitated rats using a chemical method.

Nature of Plasma-Reducing Substances Measured. There is ample evidence that normal and abnormal human urine contains material with glycogenic

TABLE 2. EFFECTS OF ACTH ON RABBIT PLASMA LIPID-SOLUBLE REDUCING SUBSTANCES

Rabbit No.[1]	Dose ACTH[3] mg./kilo	Plasma reducing value mg./%[4]		
		Before injection	2 Hrs.	4 Hrs.
1	0.1	0.70	0.59	0.42
2	0.2	0.60	0.61	0.60
3	0.4	0.78	2.74	1.17
4	0.6	0.44		1.22
5	1.6	0.53		0.84
6[2]	1.6	1.34 (venous) 1.06 (heart)		2.60 (venous) 1.47 (heart)
7	2.6	0.56	2.12	1.13
8	3.4	0.67		2.86

[1] Rabbit Nos. 1, 2, 3, 5 and 7 were non-fasted animals and Rabbit Nos. 4, 6 and 8 were fasted 3 days before use. All blood samples except as indicated in Rabbit No. 6 were obtained by heart puncture.

[2] This rabbit had been prepared for evisceration by constriction of the vena cava and had fully developed abdominal collateral circulation and hence the venous samples were taken from the abdominal veins.

[3] The ACTH was given intramuscularly. Kindly supplied by Dr. J. R. Mote of Armour and Company. Doses given are equivalent to the Armour Standard, La-1-A.

[4] 17-hydroxy-11-dehydrocorticosterone used as reference standard. Values not corrected for losses shown in Table 1.

activity similar to the 11-oxygenated adrenal cortical hormones. Mason and Sprague (1948) have isolated 17-hydroxycorticosterone from the urine in a case of Cushing's syndrome. More recently Mason (1950) reported the isolation of a substance as an acetate which had the properties of 17-hydroxycorticosterone from post-operative urine and 17-hydroxycorticosterone was isolated from the urine of patients suffering from rheumatoid arthritis who had received adrenocorticotropic hormone. The presence of 17-hydroxy-11-dehydrocorticosterone and 17-hydroxycorticosterone in the urine of normal persons has been indicated by analyses using paper partition chromatography (Zaffaroni *et al.* (1950), and Schneider (1950) has isolated 17-hydroxy-11-dehydrocorticosterone from the same source. As a result of these findings it seems most likely that the increased plasma-reducing substances observed after the administration of ACTH is a meas-

ure of the adrenal excretion of 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone.

Specificity of Method. The phosphomolybdic acid reducing method was chosen for these studies for its simplicity and reliability (Romanoff *et al.* (1949)). Wick *et al.* (1950) have shown that chemical analysis of fractionated normal urine extracts, based on the formation of formaldehyde by periodic acid oxidation, gave values in terms of corticosterone 6–8 times higher than those obtained by *in vivo* glycogen deposition. In all probability a similar ratio applies for the plasma-reducing lipids and the biological titer so that the normal values reported here for plasma-reducing lipids may include substances other than those of adrenal cortical origin. Cortin compounds may exist in the plasma in a conjugated form having different extraction characteristics than the free compounds. The method reported here does not recover 11-desoxycorticosterone-glucoside. This water soluble steroid is soluble in wet chloroform but it is removed when the chloroform is dried. The normal plasma lipid-soluble reducing equivalent will also depend on the standard chosen since the crystalline adrenal compounds possess different degrees of reducing action.[1] The use of 11-desoxycorticosterone as a standard gives values approximately 35% lower than that obtained with 17-hydroxy-11-dehydrocorticosterone. In view of the above variables no attempt is made in this report to attach any significance to the 'normal' plasma values. It is believed that the rise in the lipid-soluble reducing value observed after the administration of ACTH is more important and reflects adrenal cortex stimulation.

SUMMARY

A method has been described, based on the reduction of phosphomolybdic acid, which is suitable for determining the increase of lipid-soluble reducing substances in the plasma of rabbits after administration of adrenocorticotropic hormone. Administration of ACTH to rabbits at 0.4 mg./kilo or greater produced an increase in the plasma reducing value. Lower doses of ACTH were ineffective.

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[1] By subjecting equal quantities of the crystalline compounds to the phosphomolybdic acid color development the relative reduction may be compared as follows: 11-desoxycorticosterone, 100; corticosterone, 85; Cpd. E, 64 and DOC-glucoside, 31.

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THE EFFECT OF STEROIDS AND LACTONE DERIVATIVES ON HYPOPHYSEAL GONADOTROPHIN CONTENT[1]

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THE search for exogenously administered agents which will control pituitary gonadotrophic function has revealed that inhibition may be produced by estrogens, androgens and progesterone when large doses are given. Mild stimulation may result from administering small amounts of some of these steroids. Clinical application of these agents in instances where pituitary suppression or stimulation is desired is limited by their marked sex hormone effects. For example, it would be ideal to cut off pituitary stimulation to the ovary in endometriosis or premature puberty. However, (1) estrogens cannot be used because amounts large enough to suppress pituitary gonadotrophins aggravate these conditions; (2) androgens are limited in usefulness by their virilizing effects; and (3) the dosage of progesterone is too large to be practicable. No potent hypophyseal stimulator is known. Therefore, a number of substances unlikely to exhibit marked estrogenic or androgenic activity were screened to determine their ability to depress or stimulate hypophyseal gonadotrophic output, at the same time assessing their estrogenic activity. Androgenic potency was to be determined if a significant effect upon the pituitary was noted.

Enormous daily doses, calculated to exceed the physiological level by a wide margin, were injected into adult female rats immediately after castration to determine whether the post-castration rise in gonadotrophins was either prevented or augmented. Only marked deviations from the expected rise were considered significant in view of the large doses of each substance used. Estrogenic activity was judged by the effects on vaginal cells, uterine and thymic weights.

The compounds screened were synthetic steroids related to progesterone, pregnenolone, testosterone and corticosterone, as well as the lactones of estrone and testosterone. Water-soluble testicular extracts, alleged to contain "inhibin," were also tested.

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[2] Schering Fellow in Endocrinology.

METHOD

Adult Sprague-Dawley rats weighing between 200 and 260 gm. were surgically castrated after they were shown to have regular estrus cycles by daily vaginal smear examinations. The steroid injections, given subcutaneously every 12 hours for 16½ days, were instituted within 24 to 48 hours following castration. Daily vaginal smear examinations were conducted during treatment. At autopsy, 24 hours after the last injection, organs were weighed and the anterior pituitary glands frozen. The assay for pituitary content was carried out by the method of Jungck, Maddock and Heller (1947) on 24-day old female Sprague-Dawley rats, injecting one donor pituitary completely dispersed in 6 cc. of water, to each recipient assay rat at the rate of 1 cc. twice daily for 3 days.

DISCUSSION

Of the 14 steroids tested not one prevented the post-castration rise in gonadotrophins (Table 1). In a few instances where this rise appears to be less than the uninjected castrates, the possibility of minimal suppression could be entertained. Any significant pituitary suppression, however, is ruled out considering the large doses of steroid administered. Moreover, many more animals per dose would be required to determine a significant difference between any two groups.

As judged by daily vaginal smear examinations, none of the compounds manifested significant estrogenic activity, which was further attested to by the failure of the thymus to undergo atrophy and the uteri to markedly increase in weight.

A hormone other than androgen or estrogen, secreted by the testis and causing pituitary inhibition, has been postulated by McCullagh and Walsh (1935) and Klinefelter, Reifenstein and Albright (1942), called either "inhibin" or "X" hormone. Howard, Sniffen, Simmons and Albright (1950) made the suggestion that their theoretical "X" hormone is pregnenolone. However, the "X" hormone, if such exists, cannot be pregnenolone since both Δ^5-pregnenolone acetate and Δ^5-acetoxypregnenolone are utterly devoid of pituitary suppressing activity. Clarke and Selye (1943) have shown that 10 mg. of Δ^5-pregnenolone, given to immature female rats daily, does not prevent the appearance of pituitary castration cells. We have determined that Δ^5-pregnenonlone is unable to suppress urinary gonadotrophin excretion in man by administering 100 mg. of this steroid daily for 100 days to a normal 37 year old man.

Of all the compounds tested only the lactone of estrone, estrololactone acetate in oil, prepared by the method described by Jacobsen (1947), demonstrated any pituitary suppression worthy of note (Table 2). Suppression, while incomplete, was positive at the 1000 μg. daily level. This is in keeping with the observations of Bradbury (1947).

In comparing estrololactone to estrone it was noted that estrone caused complete pituitary suppression at one hundreth the daily dose level re-

TABLE 1. ESTROGENIC AND PITUITARY ACTIVITY OF 14 STEROIDS INJECTED INTO ADULT FEMALE CASTRATED RATS

Castrated Female Donor Rats				Anterior Pituitary Recipient Rats[3]		
Compound	Daily dose for 16½ days mg.[4]	No. of rats	Vag. cytology	Vag. open.	Ut. wt. mg.	Ovar. wt. mg.
Δ^5-pregnenolone acetate[6] (pregnenolone)	10	3	Anestrus	+	88	60
Δ^5-21-acetoxypregnenolone[6] (acetoxy pregnenolone)	10	3	Anestrus	+	101	79
Δ^4-pregnene-17(α),20,21-triol-3-one-20, 21-diacetate[6] (pregnenetriolone diacetate)	10	3	Anestrus	+	95	104
17(α)-hydroxy progesterone[6]	10	3	Anestrus	+	96	57
17(β)-hydroxy progesterone[6]	10	3	Anestrus	+	80	81
Δ^4-pregnene-17(β),20,21-triol-3-one[6] (dihydrodihydroxyprogesterone)	10	3	Anestrus	+	107	82
$\Delta^{16,17}$-dehydroprogesterone[5]	10	3	Anestrus	+	85	93
Acetoxytestosterone[5]	10	3	Anestrus	+	83	60
$\Delta^{1,4}$-androstadiene-3,17-dione[5,6]	10	5	Anestrus	+	93	52
Testololactone[7] (Jacobsen's method)	10	3	Anestrus	+	89	94
Δ^5-androstene-3(β)-ol-17-one-3-acetate[6] (dehydroisoandrosterone acetate)	10	3	Continued Proestrus	+	80	78
Androstane-3,17-dione[6] (Androstanedione)	10	3	Anestrus	+	84	86
$\Delta^{4,6}$-androstadiene-3,17-dione[5]	10	3	Anestrus	+	95	85
Reichstein's substance S[6]	10	3	Anestrus	+	89	88
Uninjected castrated controls	0	15	Anestrus	+	95	81
Uninjected intact controls	0	4	Cycling	+	138	17.3
Uninjected intact recipient controls	—	15	—	—	24	13

quired for estrololactone (10 μg. versus 1000 μg.). The minimal dose levels used causing continuous vaginal estrus were 100 μg. for estrololactone and

[3] Each recipient rat received the anterior pituitary suspension of one donor rat, injected twice daily for 3 days.

[4] Each donor rat was injected twice daily.

[5] Kindly supplied by Ayerst, McKenna and Harrison, Ltd., New York, N. Y.

[6] Kindly supplied by Schering Corp., Bloomfield, N. J.

[7] Kindly supplied by G. D. Searle and Co., Chicago, Ill.

1 μg. for estrone, a ratio of 100 to 1. Thus, estrololactone is qualitatively similar to estrone in suppressing hypophyseal function and in causing continuous vaginal estrus, but quantitatively about one hundredth as effective. This ratio of 100 to 1 suggests that our estrololactone was contaminated with the more active estrogen, estrone. On the other hand, these responses may be intrinsically characteristic of estrololactone.

Two water-soluble testicular extracts were assayed for their pituitary depressant effects. One was prepared by the method of Vidgoff, Hill, Vehrs and Kubin (1939) for "inhibin"; the other was an aqueous testicular ex-

TABLE 2. ESTROGENIC AND PITUITARY ACTIVITY OF ESTRONE AND ESTROLOLACTONE INJECTED INTO ADULT FEMALE CASTRATED RATS

	Castrated Female Donor Rats						Anterior Pituitary Recipient Rats		
Compound	Daily dose for 16½ days μg[1]	No. of rats	Vag. cytology	Thymus wt. mg.	Uterine wt. mg.	Ant. pit. wt. mg.	Vag. open.	Ut. wt. mg.	Ovar. wt. mg.
Estrololactone[7] (Jacobsen's method)	10	3	Anestrus	665	136	9.4	+	130	88
	100	4	Estrus	544	245	9.8	+	88	74
	1000	4	Estrus	250	442	10.6	+	114	45
Estrone (Theelin)	1	4	Estrus	380	410	11.3	+	97	102
	10	3	Estrus	152	667	14.0	−	75	17
	100	4	Estrus	74	613	13.8	−	48	12
	200	4	Estrus	82	690	14.6	−	41	10
Uninjected Castrated Controls	0	15	Anestrus	480	147	9.5	+	95	81
Uninjected Intact Controls	0	4	Cycling	245	398	9.0	+	138	17.3
Uninjected Intact Recipient Controls	—	15	—	—	—	—	−	24	13

tract marketed as "Androstine."[8] Ten and 25 gm. equivalents were injected daily for 10 days without preventing post-castration rise in gonadotrophins. Neither exerted any estrogenic activity (Jungck and Maddock, 1948).

It is possible, since all known steroids capable of significantly altering pituitary content concomitantly exert sexual manifestations, that these two effects are but dual manifestations of a single aspect of steroid activity. Noble, Plunkett and Taylor (1950) observed inhibition of pituitary content in rats given Lithosperm, noting no appreciable secondary sexual activity. This substance, however, appears to be toxic* and the suppression is easily explained on this basis.

[8] Kindly supplied by CIBA Pharmaceutical Products, Inc., Summit, N. J.

* Since this article was written there has appeared evidence of a non-toxic preparation of Lithosperm.

SUMMARY

In search for compounds capable of stimulating or depressing pituitary gonadotrophic output without exerting secondary sexual effects, 16 miscellaneous steroids and 2 testicular extracts were administered to female adult castrated rats for periods of 10 and 16½ days and pituitary potency assessed.

None of the 14 steroids listed in Table 1 manifested significant pituitary depressing or stimulating effects or estrogenic activity.

Both estrone and estrololactone were shown to have pituitary suppressant and estrogenic activity in comparable dose ratios, estrololactone, however, being 100 times less potent.

Two testicular extracts given in daily doses equivalent to 10 and 25 gm. of whole testis likewise exerted no effect upon the hypophysis.

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STUDIES WITH LABELLED ANTERIOR PITUITARY PREPARATIONS: ADRENOCORTICOTROPIN

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THE determination of the *in vivo* effects of the anterior pituitary hormones has depended largely upon classical endocrinological techniques. These have included hypophysectomy with or without hormone replacement as well as the direct administration of the hypophyseal hormones to normal as well as prepared animals. The fate of these protein hormones has not been adequately elucidated with such methods. It was considered of interest to study the distribution of anterior pituitary hormone preparations after being labelled with different radioactive reagents.

Many investigators have reported that such procedures as the coupling of a diazonium compound to protein, or the direct iodination of protein may alter the protein's previous specificity and possibly lead to the acquisition of new immunologic properties (Landsteiner, 1945, Haurowitz, Sarafian and Schwerin, 1941, Shahrokh, 1943). The average number of substituent groups introduced (Landsteiner, 1945, Haurowitz, Sarafian and Schwerin, 1941, Shahrokh, 1943) as well as the role played by the substituted amino acid in determining the specificity of the protein molecule (Landsteiner, 1945) will influence the degree to which new properties are acquired. Iodination may also lead to the oxidation of sulfhydryl groups and other oxidations which may partly determine the degree of loss of biological activity. There is now a considerable body of evidence which points to the conclusion that when some proteins are labelled, with only one or two substituent groups per protein molecule, their biological activity may not be measurably affected. Thus hen's egg albumin treated with ten azo groups per molecule is only 50 per cent less reactive immunochemi-

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cally than the unlabelled or lightly labelled preparation (0.67 groups per protein molecule) (Francis and Wormall, 1948). Insulin coupled with 6 azo groups per protein molecule maintained its activity almost unchanged (Reiner and Lang, 1941).

In recent years some proteins of biological importance have been labelled with radioactive iodine, e.g. bovine serum albumin (Eisen and Keston, 1948 and 1949) and antibody preparations (Pressman and Keighley, 1948, Pressman, Hill and Foote, 1949, Pressman, 1949, Pressman, 1949a, Pressman, 1949b, Pressman and Eisen, 1950). Insulin preparations labelled by reacting with radioactive diazotised p-iodoaniline have been employed in the studies of the absorption of insulin from sites of injection (Reiner, Keston and Green, 1942).

The technique of trace labelling (Eisen and Keston, 1948 and 1949) in which the protein is labelled with a small fraction of radioactive substituent molecules per protein molecule would seem to offer the highest probability of labelling a protein with minimal loss of biological activity. When such small tracers of radioactive substituent molecules are used, it becomes very improbable that the protein molecule which has been labelled has been oxidized or undergone chemical reaction in any other way. Measurement of the biological activity of the trace labelled preparation gives no clue as to whether the labelled molecules have still retained their biological activity since the preparation consists mostly of the original unreacted biologically active molecules. To determine whether the labelled molecules are active it is necessary, in the case of a homogeneous protein, to study how the biological activity decreases as larger amounts of substituent molecules are employed. For a non-homogeneous protein preparation it is necessary, in addition, to know the kinetics of labelling each constituent, the rates of which may vary widely (Pressman and Sternberger, 1950).

Anterior pituitary hormone preparations with adrenocorticortophic activity (Armour ACTH) labelled by iodination with traces of radioactive iodine (I^{131}) were employed in this study. Li, Simpson, and Evans, (1946), have shown that such hormone preparations retain considerable activity after treatment with several non-radioactive iodine molecules per molecule of protein. Since the ACTH may not be a homogeneous protein and only a small amount of the preparation may be the active substance, the data on the biological activity after iodination must be considered as inconclusive. ACTH has been prepared in a much more active form (Chem. and Eng. News, 1950. Astwood, Raben, Payne and Cleroux, 1950) than the preparations studied earlier by these investigators. However, it is possible to state that under conditions where the bulk of the protein present in an ACTH preparation had been iodinated with several iodines per molecule

of protein, considerable biological activity remained (Li, Simpson and Evans, 1946).

EXPERIMENTAL

A. Chemical

Approximately 15 milligrams of an adrenocorticotrophic hormone preparation (Armour ACTH)* were weighed out and dissolved in 0.1 cc. of a 1 molar solution of sodium carbonate-sodium bicarbonate, pH 8.8. Five different lots of Armour ACTH with varying biological potency and degree of contamination with other pituitary hormones have been used in this study. (The correlation between potency and localization of the labelled preparation will be reported in a future publication.)

In a small test tube 20 millicuries of carrier-free I^{131} were evaporated to dryness on a water bath under a stream of nitrogen. To this was added in turn 0.1 cc. 0.002M KI, 0.1 cc. 0.15M $NaNO_2$, and 0.1 cc. 0.18N H_2SO_4. The excess nitrous acid was destroyed with 0.1 cc. 1M ammonium sulfamate. The solution was made alkaline with 0.1 cc. of Na_2CO_3-$NaHCO_3$ buffer and added to the dissolved ACTH preparation. This was allowed to remain at room temperature for one hour and then transferred to a Visking dialysis tube. A minimal quantity of this mixture was retained for precipitation studies. The preparation was dialysed in the cold (4°C.) with motor driven stirring against four 17 liter changes of isotonic saline over two days. (There were only traces of radioactive iodine in the third dialysate and no radioactivity could be determined in the fourth dialysate.) It was not determined whether the dialysates contained iodinated protein fragments as well as free iodine. Less than 0.01% of the radioactivity contained in the dialysis bag was present in the fourth dialysate. (Approximately 7 per cent of the radioactivity remained after dialysis.) The contents were brought to a concentration of 1 mg. of ACTH preparation per cc. for injection purposes.

To the small amount of reaction mixture retained was added 0.1 cc. of 1M $Na_2S_2O_3$ and 1 cc. of 0.02M KI. Approximately 5 milligrams of ACTH as carrier were included in this mixture and an equal volume of 10% trichloracetic acid was added. The preparation was centrifuged, supernatant removed, and the precipitate washed two times with 5% CCl_3COOH. Counts were determined in the total precipitate fraction and supernatant in order to determine the per cent radioactivity on the protein. (7 to 10 per cent of the radioactivity was found in the trichloracetic acid precipitate. Higher initial concentrations of iodine did not alter this per cent of iodination.)

B. Biological

Two hundred and fifty normal adult male Sprague-Dawley rats were used in this study. (All the experiments are not tabulated in this report. The data reported, however, is representative of all the results.) These were injected intracardially under nembutal anesthesia (9 mg. per rat) and were sacrificed at various intervals of time. Each injection contained between 0.2 mg. and 3.2 mg. of the ACTH preparation labelled with 100 microcuries of I^{131}. The animals were killed by exsanguination and portions of representative organs (thyroid, adrenal, gonads, pancreas, muscle, thymus, liver, spleen, kidney, and blood) were immediately removed, weighed, and placed in specimen vials containing Bouin's Solution.

The method of counting samples was that of taking 10 ml. vials containing either the

* We wish to thank Dr. John R. Mote, Armour Laboratories, for the ACTH preparations used in this study.

tissue or a standard solution consisting of a known dilution of the original material. These were placed in the center of a multiple center wire gamma ray Geiger tube (Marinelli and Hill, 1948a). In our experience the over-all error due to positioning and statistics could not be greater than 2%. The results have been expressed as the fraction of the injected material which localizes per gram of tissue.

The specimens were then imbedded in paraffin for histological sections and radioautography according to the technique of Marinelli and Hill (1948b). Similar experiments with the same amount of iodide ion labelled with I^{131} and I^{131} labelled bovine serum albumin were carried out. Control radioautographs of similar tissues from animals not given radioactive substances were made to exclude the possibility of chemoreduction of the photographic emulsion.

RESULTS

A. Time Curves

Shortly after the administration of an ACTH preparation labelled with I^{131}, radioactivity was found in significant concentrations in the adrenals, blood, liver, spleen, kidney, and later in the experiment in the thyroid (Tables 1 and 2). The concentrations of radioactivity in these organs were much greater than in the other removed organs. The concentration fell very rapidly in all organs except the thyroid (Figures 1 to 5) so that little radioactivity could be detected after four hours. The concentration of radioactivity in the adrenals and other organs was affected only slightly by the amounts, from 200 to 3200 gamma (Figure 6) of ACTH preparations which were labelled with I^{131}. In all experiments, the concentration in the thyroid increased as the concentrations in the other organs decreased (Figure 4).

Following the administration of a radio-iodinated bovine serum albumin preparation with the same amount of iodine injected per animal as with ACTH·I^{131}, the greatest degree of radioactivity per gram of tissue was noted in the liver, spleen, blood and kidney (Table 3). There was significantly less radioactivity in the adrenal (Figure 1) when compared to the ACTH·I^{131} experiment. After four hours, there was little reduction in the concentration of I^{131} in most of the organs (Figures 1, 2, 3, and 5, Table 3). During the same period of time there was a much smaller increase in the thyroid concentrations (Figure 4) than was noted with ACTH·I^{131} or I^{131} alone.

When the same amount of iodide (about 0.1 gamma per animal) with I^{131} was given there was little concentration in any organ other than the thyroid which showed a prompt increase as the blood concentration fell (Table 4).

Iodinated ACTH was prepared with an iodine to protein ratio of 1:60 and another preparation with a ratio of 2:1. To determine these ratios the assumption was made that we had a homogeneous protein of average molecular weight of 20,000 (Li and Evans, 1947). Whether the differences

TABLE 1. RELATIONSHIP BETWEEN TIME AND CONCENTRATION OF RADIOACTIVITY AFTER THE ADMINISTRATION OF AN I^{131} LABELLED ACTH PREPARATION. (ARMOUR LOT NUMBER G-1012.) EACH ANIMAL RECEIVED 1000 GAMMA OF PROTEIN LABELLED WITH 0.07 GAMMA OF IODINE, CONTAINING 100 μC. OF I^{131}.

ACTH·I^{131} No. 3									
Time	Rat wt. gms.	Thyroid wt. mg.	Counts per min.	% per sample	% per gm.	Adrenal wt. mg.	Counts per min.	% per sample	% per gm.
30 sec.	320	16.9	608	.018	1.06	40.0	1298	.037	.925
1 min.	320	15.1	768	.022	1.45	34.4	983	.028	.813
	315	16.5	518	.015	.91	32.3	878	.025	.774
	338	18.8	568	.016	.85	39.2	1138	.033	.842
2 min.	315	21.3	1048	.030	1.41	34.2	643	.018	.526
	317	15.2	708	.020	1.32	36.3	1198	.036	1.000
	347	17.3	1418	.041	2.37	45.0	938	.027	.600
4 min.	302	17.3	2288	.066	3.81	40.3	808	.023	.570
	336	13.2	1198	.035	2.67	40.2	798	.023	.570
	265	15.1	2208	.063	4.17	33.7	698	.020	.593
8 min.	325	15.0	3498	.101	6.74	37.5	613	.018	.480
	347	19.8	4888	.140	7.06	45.8	603	.017	.371
	305	17.3	3308	.095	5.55	37.3	508	.015	.402
15 min.	283	15.2	7338	.210	13.8	34.4	488	.014	.407
	337	17.8	11830	.398	22.3	48.4	553	.016	.331
	345	17.4	15488	.445	25.5	39.0	373	.010	.256
30 min.	348	17.9	20888	.602	33.6	39.9	403	.012	.300
	298	17.8	20048	.576	32.4	30.1	283	.008	.266
	345	18.5	46088	1.30	70.3	35.0	291	.009	.257
1 hr.	332	15.6	49288	1.42	91.0	44.0	266	.008	.181
	325	20.3	56088	1.62	78.8	35.2	233	.007	.199
	305	18.1	68088	1.96	108.1	41.1	274	.008	.195
2 hr.	340	14.5	71288	2.05	141.2	31.4	133	.004	.127
	340	17.6	102000	2.94	167.0	41.6	168	.005	.120
	300	18.3	85188	2.45	133.9	35.1	148	.004	.114
4 hr.	340	21.1	192000	5.54	262.3	30.5	54	.002	.066
	293	17.0	132000	3.80	223.8	41.2	167	.005	.121
		Blood vol. cc.				Muscle wt. mg.			
30 sec.	320	5.0	55460	12.550	2.51	39.1	138	.004	.102
1 min.	320	5.0	55137	12.500	2.50	87.5	598	.016	.183
	315	5.0	56257	12.800	2.56	89.8	368	.011	.122
	338	5.0	56017	12.700	2.54	51.5	173	.005	.097
2 min.	315	5.0	31737	7.200	1.44	112.5	98	.002	.178
	317	5.0	44047	10.050	2.01	77.3	262	.008	.103
	347	1.0	5108	1.160	1.16	109.9	558	.016	.146
4 min.	302	5.0	25203	5.700	1.14	89.1	368	.011	.123
	336	5.0	26203	5.950	1.19	174.7	683	.020	.114
	265	5.0	32737	7.400	1.48	189.7	1138	.033	.173
8 min.	325	5.0	17047	3.885	.777	231.9	968	.028	.121
	347	5.0	16513	3.750	.750	220.0	753	.022	.100
	305	5.0	20747	4.700	.940	74.1	423	.012	.162

Time	Rat wt. gms.	Blood vol. cc.	Counts per min.	% per sample	% per gm.	Muscle wt. mg.	Counts per min.	% per samplé	% per gm.
15 min.	283	5.0	18097	4.100	.820	143.7	618	.018	.125
	337	5.0	15717	3.575	.715	72.8	273	.008	.110
	345	5.0	13382	3.150	.630	114.0	441	.013	.114
30 min.	348	5.0	14082	3.200	.640	172.4	668	.020	.116
	298	5.0	14067	3.200	.640	56.3	273	.008	.142
	345	5.0	13157	2.985	.597	106.5	132	.004	.038
1 hr.	332	5.0	11477	2.610	.522	66.0	238	.007	.106
	325	5.0	11787	2.675	.535	102.8	453	.013	.126
	305	5.0	10258	2.325	.465	209.0	638	.019	.091
2 hr.	340	5.0	7966	1.815	.363	68.7	153	.004	.058
	340	5.0	7653	1.740	.348	46.2	113	.003	.065
	300	5.0	6592	1.500	.300	61.8	128	.004	.065
4 hr.	340	5.0	6408	1.455	.291	71.9	103	.003	.042
	293	5.0	7857	1.560	.312	130.2	403	.011	.084

in the extent of localization of radioactivity noted in the adrenal with these preparations is significant has not been ascertained (Figure 6).

Complete studies of the biological activity of our radio-iodinated ACTH preparations have not been done. Preliminary data of such a study of the more heavily iodinated (2:1) preparation reveal retention of biological activity. Preparations only minimally labelled, e.g. 1:60 would not be expected to have significant change in biological activity since only a trivial percentage of the total number of protein molecules could have reacted with the labelling reagent. The biological activity of such preparations has not been tested.

B. Radioautography

Only the adrenal and thyroid glands showed positive radioautographs. These reflected the time curves as determined by direct radioactivity measurements. Although there was exposure throughout the entire adrenal there was considerably more in the cortex with a definite preference for the inner layers (Figure 7). The radioautographic representation was in no way altered by larger amounts of protein injected, or amounts of iodine up to two iodine molecules per protein molecule. In no case with the radioactive ACTH preparation could a positive radioautograph be obtained with other tissues except the thyroid. Control studies with inorganic radioactive iodide and radio-iodinated bovine serum albumin gave no positive radioautographs either in the adrenal or in the other organs, except the thyroid. The thyroid radioatuographs were similar to those obtained when inorganic radioactive iodide was given, Radioautographs of adrenals from animals receiving no radioactivity showed no reduction of the photographic emulsion. This excluded the possibility of chemical reduction of the photographic emulsion by adrenal tissue.

TABLE 2. RELATIONSHIP BETWEEN TIME AND CONCENTRATION OF RADIOACTIVITY AFTER THE ADMINISTRATION OF AN I^{131} LABELLED ACTH PREPARATION. (ARMOUR LOT NUMBER J9401-A.) EACH ANIMAL RECEIVED 3200 GAMMA OF PROTEIN LABELLED WITH 0.2 GAMMA OF IODINE, CONTAINING 100 μC. OF I^{131}.

ACTH·I^{131} No. 8

Time	Rat wt. gms.	Thyroid wt. mg.	Counts per min.	% per sample	% per gm.	Adrenal wt. mg.	Counts per min.	% per sample	% per gm.
1 min.	180	3.5	605	.013	3.71	13.5	1315	.016	1.20
	230	17.4	1345	.023	1.32	31.8	2635	.032	1.01
4 min.	163	11.9	2326	.039	3.28	33.8	3355	.041	1.21
	188	12.7	2488	.043	3.39	32.3	2195	.027	.835
15 min.	215	11.1	20085	.342	30.8	35.2	1025	.013	.369
	225	14.8	8235	.139	9.4	32.7	870	.011	.336
1 hr.	187	12.4	15525	.264	21.2	31.9	419	.005	.157
	190	11.0	31975	.542	49.2	27.0	745	.009	.334
2½ hr.	160	—	—	—	—	32.7	767	.009	.275
	210	13.3	73125	1.24	93.5	31.8	435	.005	.157
		Blood vol. cc.				Kidney wt. mg.			
1 min.	180	1.0	8595	5.30	5.30	62.3	42350	.945	14.9
	230	1.0	7271	4.50	4.50	97.3	22150	4.95	51.0
4 min.	163	1.0	7059	4.35	4.35	55.2	18795	4.20	76.2
	188	1.0	6518	4.03	4.03	218.6	55275	12.4	56.7
15 min.	215	1.0	1430	.88	.88	84.0	44700	10.0	111.0
	225	1.0	1390	.86	.86	95.4	31775	7.1	71.0
1 hr.	187	1.0	610	.38	.38	50.7	2563	.57	11.1
	190	0.5	.800	.50	1.00	121.3	18795	4.20	34.5
2½ hr.	160	1.0	1555	.96	.96	146.4	31300	7.02	48.0
	210	1.0	1060	.65	.65	76.0	4205	.90	11.9
		Liver wt. mg.				Spleen wt. mg.			
1 min.	180	48.2	4615	.057	1.180	34.3	805	.010	.291
	230	145.9	12515	.154	.969	55.7	2207	.027	.485
4 min.	163	60.0	7095	.088	1.465	34.6	2185	.027	.730
	188	21.1	2115	.026	1.211	37.3	1645	.020	.536
15 min.	215	55.0	2095	.026	.473	65.0	1755	.022	.339
	225	56.5	1955	.024	.426	69.7	1730	.021	.301
1 hr.	187	36.6	475	.006	.164	49.1	520	.006	.122
	190	96.0	2985	.037	.385	50.3	1495	.018	.358
2½ hr.	160	58.4	1675	.021	.360	50.8	1605	.020	.393
	210	72.1	1265	.016	.222	50.7	943	.017	.335
		Muscle wt. mg.				Thymus wt. mg.			
1 min.	180	62.9	1165	.014	.222	104.0	1995	.025	.235
	230	93.4	878	.011	.118	68.6	1365	.017	.248

Time	Rat wt. gms.	Muscle wt. mg.	Counts per min.	% per sample	% per gm.	Thymus wt. mg.	Counts per min.	% per sample	% per gm.
4 min.	163	84.7	1365	.017	.202	41.7	645	.008	.191
	188	58.0	720	.009	.155	62.1	545	.007	.112
15 min.	215	65.3	1115	.014	.214	—	—	—	—
	225	50.5	655	.008	.158	58.5	535	.007	.120
1 hr.	187	148.1	1075	.013	.083	38.8	1335	.016	.413
	190	99.8	1705	.021	.211	66.9	585	.007	.105
2½ hr.	160	65.4	945	.012	.183	51.4	1805	.022	.427
	210	77.2	657	.008	.103	68.2	1020	.013	.191

DISCUSSION

There are several explanations as to why a greater degree of localization was not noted in the adrenal with a supposedly biologically specific material like ACTH. Firstly, if our preparations were but one per cent pure in terms of ACTH activity, we would be faced with the situation where ninety-nine per cent of the injected material would be non-specific protein whose distribution would completely mask that of the biologically specific one per cent. The high concentrations of the labelled ACTH preparation in the liver and spleen which are part of the reticuloendothelial system may be in part the result of the action of these two organs on non-specific protein. Secondly, the limited blood flow through the adrenal would greatly limit the amount of ACTH preparation which could be cleared by the adrenal. Thirdly, in our estimations we have included the biologically inactive (with reference to ACTH) adrenal medulla. Fourthly, it seems quite reasonable that ACTH may localize and disappear very rapidly from the adrenal cortex. Adrenal ascorbic acid is lowered within thirty minutes after subcutaneous injection in the rat (Sayers and Sayers, 1949). Inasmuch as our ACTH preparations were administered intracardially, we might anticipate a more rapid biological effect. Circulating blood eosinophiles have been noted in man to be lowered within twenty minutes after subcutaneous injection of ACTH (Thorn, *et al.*, 1948). ACTH has been shown (Greenspan, Li and Evans, 1950) to have a half time of disappearance from rat's plasma of 5.5 minutes which is about the same order of time in which the radioactivity in the adrenal diminished to a half of the maximum value recorded (Figure 1). Finally, it would appear that the dosage of ACTH used was excessive, being 200 to 3200 times the amount necessary to cause maximum adrenal ascorbic acid depletion. The introduction of doses more closely approximating the physiologic range might result in different localization patterns. This problem is being investigated.

The possibility must be considered that the radioactivity found in the

TABLE 3. RELATIONSHIP BETWEEN TIME AND CONCENTRATION OF RADIOACTIVITY AFTER THE ADMINISTRATION OF AN I^{131} LABELLED PREPARATION OF BOVINE SERUM ALBUMIN (BSA). (ARMOUR LOT NO. J4902.) EACH ANIMAL RECEIVED 1000 GAMMA OF PROTEIN LABELLED WITH 0.1 GAMMA OF IODINE, CONTAINING 100 μC. OF I^{131}.

BOVINE SERUM ALBUMIN I^{131} No. 6

Time	Rat wt. gms.	Thyroid wt. mg.	Counts per min.	% per sample	% per gm.	Adrenal wt. mg.	Counts per min.	% per sample	% per gm.
1 min.	200	7.4	53	.0016	.216	17.3	127	.004	.231
4 min.	200	15.0	134	.0040	.266	34.9	280	.0084	.240
	215	13.5	66	.0019	.140	35.1	232	.0070	.199
15 min.	237	17.1	77	.0023	.134	29.1	192	.0058	.199
	188	11.9	118	.0035	.294	33.5	203	.0061	.182
1 hr.	234	19.3	254	.0076	.394	31.4	122	.0037	.119
	208	12.3	417	.0125	1.02	25.4	132	.0039	.153
2½ hr.	205	14.0	577	.0173	1.24	33.9	224	.0067	.197
	190	11.4	1757	.0526	4.64	33.5	152	.0046	.137
		Blood vol. cc.				Kidney wt. mg.			
1 min.	200	1.0	62700	1.88	1.88	95.8	867	.0260	.272
4 min.	200	1.0	69100	2.06	2.06	91.3	1167	.0350	.384
	215	1.0	51200	1.54	1.54	63.7	557	.0167	.262
15 min.	237	1.0	41600	1.35	1.35	89.1	632	.0190	.213
	188	1.0	54900	1.65	1.65	71.7	697	.0209	.292
1 hr.	234	1.0	25900	0.67	0.67	87.1	462	.0138	.158
	208	1.0	48900	1.47	1.47	56.8	392	.0118	.208
2½ hr.	205	1.0	34250	1.02	1.02	82.4	427	.0129	.157
	190	1.0	34750	1.04	1.04	70.3	357	.0107	.152
		Liver wt. mg.				Spleen wt. mg.			
1 min.	200	46.7	447	.0130	.278	52.2	204	.0061	.117
4 min.	200	34.6	300	.0090	.260	25.3	373	.0112	.443
	215	64.1	369	.0110	.172	99.8	587	.0176	.177
15 min.	237	70.9	289	.0087	.123	54.4	192	.0057	.105
	188	34.1	169	.0051	.149	36.1	195	.0059	.164
1 hr.	234	66.5	247	.0074	.111	53.8	172	.0052	.097
						27.8	132	.0040	.144
2½ hr.	205	88.1	369	.0111	.126	51.3	172	.0052	.101
	190	59.6	252	.0075	.126	40.7	163	.0049	.120
		Muscle wt. mg.				Thymus wt. mg.			
1 min.	200	53.8	47	.0014	.030	103.7	103	.0031	.030
4 min.	200	65.2	82	.0024	.037	60.2	101	.0030	.050
	215	45.2	97	.0029	.064	108.8	152	.0046	.042

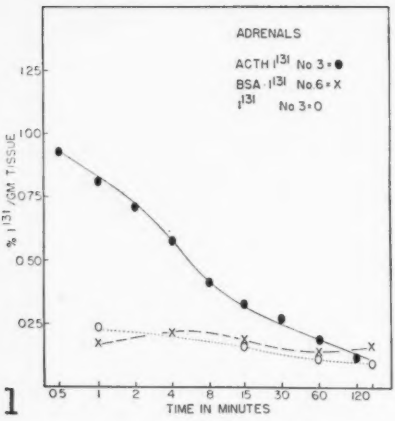
Time	Rat wt. gms.	Muscle wt. mg.	Counts per min.	% per sample	% per gm.	Thymus wt. mg.	Counts per min.	% per sample	% per gm.
15 min.	237	92.7	54	.0016	.017	102.4	139	.0041	.040
	188	42.8	25	.0008	.019	47.4	32	.0009	.019
1 hr.	234	62.0	43	.0013	.021	52.6	37	.0011	.029
	208	78.2	119	.0036	.046	75.2	83	.0025	.033
2½ hr.	205	81.7	125	.0037	.045	74.6	213	.0064	.086
	190	41.1	62	.0019	.046	61.7	37	.0011	.018

adrenal is from a constituent, in the inhomogeneous preparation, other than the adrenal cortex stimulating factor.

The possibility that the time curves for each organ and particularly the adrenal does not really reflect a true metabolism of the labelled ACTH preparation, but rather a difference in blood supply between the various organs is answered in part by the control experiments. Labelled bovine serum albumin and inorganic radioactive iodide did not show the same degree of concentration in the adrenal as did the ACTH preparation. This suggests a more specific effect for labelled ACTH. It is not likely that the disappearance curves can be related to the metabolism by one particular organ alone. The degree of localization in the adrenals was not more than 0.1% at one particular time. Although over a four hour period more than 0.1% of the administered dose might be metabolised, it does not appear reasonable that the adrenal could account for the bulk of the ACTH·I^{131} breakdown. These time curves may well represent the simultaneous phenomena of labelled ACTH destruction by adrenal and other tissues, the metabolism of free radioactive iodine and the metabolism of iodinated non-specific protein as well. The thyroid curves suggest uptake of inorganic iodide after degradation of the iodinated protein molecules.

The determination of the biological activity of a protein hormone which has had introduced different substituent groups, is of particular interest. The more heavily labelled preparation, i.e. two iodine atoms per protein molecule, has been iodinated to a much lesser degree than was found (Li, Simpson and Evans, 1946) necessary to inactivate an ACTH preparation. In general, it is conceivable that the end organ stimulating powers may be lost with retention of the localizing potential for the particular tissue.

The radioautographic results found in this study correlate well with other published biological investigations (Greep and Deane, 1949). These authors report that ACTH administration in the rat results in proliferation of the inner two layers of the adrenal cortex, the zona reticularis and the zona fasciculata. Conversely, hypophysectomy in the rat results in an atrophy of these same two layers with little effect on the zona glomerulosa.



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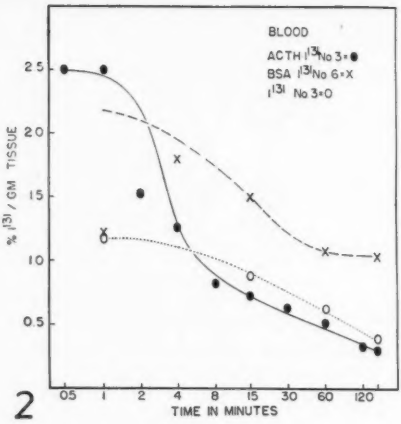

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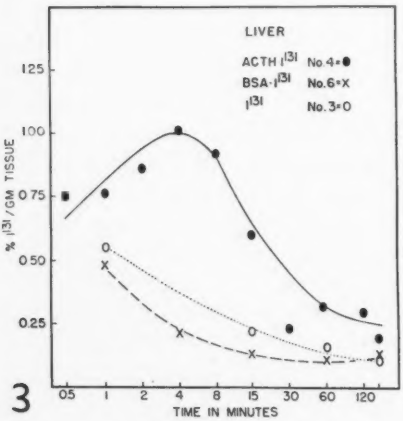

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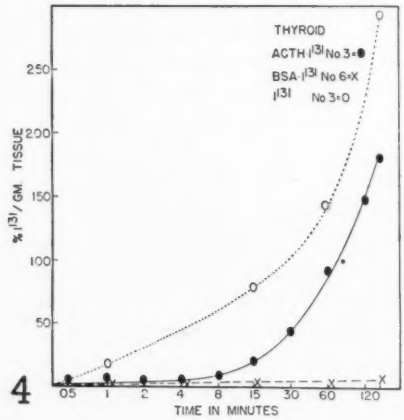

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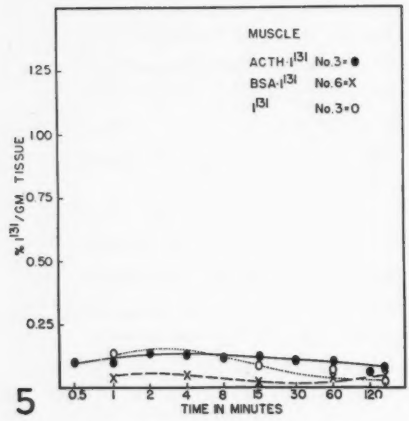

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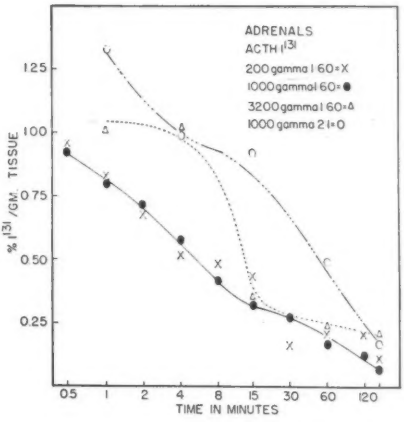
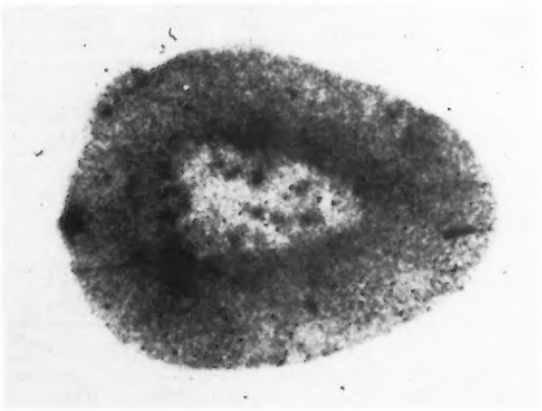
Relationship of time and concentration of radioactivity in various tissues after the administration of I^{131} labelled ACTH, I^{131} labelled bovine serum albumin (BSA), and I^{131} labelled iodide ion. Semilogarithmic scale.

It is extremely interesting to note the absence of positive radioautographs in tissues other than the adrenal and thyroid glands despite the presence of radioactivity before fixation and sectioning of the tissues. This suggests a specific binding between the constituents of the gland and constituents in the hormone preparation. The unbound components in the



Relationship of time and concentration of radioactivity in the adrenals after the administration of varying amounts of I^{131} labelled ACTH and different degrees of labelling of ACTH preparations. Semi-logarithmic scale.



Radioautograph of adrenal gland removed four minutes after the administration of an I^{131} labelled ACTH preparation. This is only the photographic emulsion without tissue superimposition.

radioactive preparation may have been lost in fixation but we have been unable to establish this as yet.

It would appear that a hormone preparation with adrenocorticortrophic activity can be successfully labelled with I^{131}. This technique has been extended to other pituitary hormones and data of considerable biological interest have been obtained. They will be reported in a future publication.

TABLE 4. RELATIONSHIP BETWEEN TIME AND CONCENTRATION OF RADIOACTIVITY AFTER THE ADMINISTRATION OF RADIOACTIVE IODIDE (I^{131}). EACH ANIMAL RECEIVED 0.12 GAMMA OF IODIDE, CONTAINING 100 μC. OF I^{131}.

	I^{131} No. 3								
Time	Rat wt. gms.	Thyroid wt. mg.	Counts per min.	% per sample	% per gm.	Adrenal wt. mg.	Counts per min.	% per sample	% per gm.
1 min.	290	15.9	23750	.530	35.4	43.4	650	.013	.300
	280	20.3	2330	.0478	2.35	18.8	170	.0034	.180
15 min.	258	15.6	37900	.845	54.2	37.3	320	.0066	.176
	300	10.4	53000	1.09	105.0	32.1	305	.0063	.196
1 hr.	272	16.0	97600	2.18	136.0	38.0	310	.0064	.168
	282	16.7	116000	2.58	155.0	38.0	215	.0044	.116
2¾ hr.	285	12.0	178500	3.97	331.0	42.0	225	.0046	.110
	283	15.6	194000	4.33	277.0	34.5	144	.0030	.087
		Blood vol. cc.				Kidney wt. mg.			
1 min.	290	1.0	5824	1.73	1.73	85.1	4552	.101	1.180
	280	1.0	2187	0.65	0.65	64.6	1070	.022	.340
15 min.	258	1.0	3003	0.890	0.890	46.0	1140	.025	.550
	300	1.0	2865	0.850	0.850	52.8	1075	.022	.420
1 hr.	272	1.0	1880	0.560	0.560	105.7	1560	.035	.331
	282	1.0	2310	0.680	0.680	72.4	780	.017	.235
2¾ hr.	285	1.0	1295	0.385	0.385	22.5	137	.003	.134
	283	1.0	1305	0.389	0.389	114.0	1020	.021	.184
		Liver wt. mg.				Spleen wt. mg.			
1 min.	290	17.9	340	.0076	.424	43.2	890	.0182	.422
15 min.	258	53.9	510	.0113	.210	43.8	480	.0099	.226
	300	37.4	428	.0088	.235	31.4	465	.0095	.304
1 hr.	272	57.5	490	.0109	.190	17.3	166	.0034	.198
	282	48.4	245	.0055	.114	24.8	140	.0029	.117
2¾ hr.	285	67.7	375	.0083	.123	31.0	210	.0043	.139
	283	83.0	417	.0090	.108	68.8	480	.0098	.143
		Muscle wt. mg.				Thymus wt. mg.			
1 min.	290	29.5	155	.0035	.119	61.3	860	.0190	.310
15 min.	258	29.9	108	.0024	.081	36.7	238	.0053	.145
	300	80.3	433	.0090	.112	35.3	398	.0082	.232
1 hr.	272	36.8	108	.0024	.065	29.6	263	.0058	.196
	282	72.1	145	.0032	.045	54.9	390	.0087	.159
2¾ hr.	285	47.5	116	.0025	.053	58.0	315	.0070	.121
	283	110.0	270	.0050	.045	55.9	372	.0076	.136

SUMMARY

An anterior pituitary preparation with adrenocorticotrophic hormonal activity has been labelled with I^{131}. The rapid entrance and disappearance of radioactivity in the adrenal has been noted after administering this preparation. Radioautographs were positive only for the adrenal and thyroid glands and indicated concentration in the inner layers of the adrenal cortex. Studies are in progress employing labelled pituitary hormones as a physiological tool.

ACKNOWLEDGMENTS

We are indebted to Dr. Rulon W. Rawson for his cooperation and enthusiastic interest in this investigation. We are also grateful to Dr. Wendell C. Peacock and Dr. John T. Godwin for their assistance.

Mr. Leon Kraintz, Miss Lorraine Kirschner, Miss Priscilla Merrill, and Miss Eva Simmel contributed valuable technical assistance in these studies.

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EFFECTS OF REPEATED INJECTIONS OF THE STEROIDS AND RELAXIN ON THE SYMPHYSIS PUBIS OF THE GUINEA PIG AS STUDIED BY X-RAY[1]

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IT HAS been known for some time that relaxation of the symphysis pubis of the guinea pig could be brought about by administration of several of the steroids and of extracts containing relaxin either singly or in combination. These studies can be summed up briefly by saying that estrogen, if given in large enough quantities and for a sufficient period of time, is able to produce separation of the pubes; that relaxin, if administered to an estrogen-primed animal, will cause within a six hour period a separation of the symphysis which is proportional to the dosage; and that progesterone can indirectly cause relaxation of the symphysis by initiating the production of relaxin from the estrogen-primed uterus. For details of this work see Hisaw *et al.* (1944), Talmage (1947), and Zarrow (1948).

In all these studies, the criterion for determining differences in the ability of the various substances to cause relaxation of the guinea pig symphysis was the time-dosage relationship at which palpable separation of the bones was obtained. Since it did not seem likely that this process of relaxation or separation was an all or none mechanism, it was desirable to study the process by x-ray whereby frequent quantitative measurements could be taken in an endeavor to better understand the process or processes of symphyseal relaxation in the guinea pig. With this in mind the following work was undertaken.

MATERIALS AND METHODS

The animals used in this study were all young female guinea pigs which had been castrated at weights between 250 and 300 grams and used in these experiments after a post-operative weight increase of at least 100 grams had been attained. In cases where hysterectomies were performed, the uteri were removed simultaneously with the ovaries. All x-rays were taken with an ordinary dental x-ray machine while the animals were under ether anesthesia. Measurements were made by means of a calibrated eye-piece in a dissecting microscope. For purposes of x-raying, the hind legs of the guinea pigs were held in position with suspended 100 gram weights. The four hormonal preparations used were as follows:

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[1] Aided in part by a grant from the Atomic Energy Commission.

(1) Estradiol[2] administered subcutaneously in sesame oil; all animals so treated were given daily doses of 1.0 μg.
(2) Progesterone administered subcutaneously in sesame oil; all animals so treated were given 1 mg. daily.
(3) Relaxin[3] administered in four equal doses each day; the total daily dose in each case was 10 G.P.U. unless otherwise specified.
(4) Pregnant Rabbit Serum obtained from a rabbit during the 28th day of pregnancy and administered in four equal doses each day, the total daily dose being 10 G.P.U.

The entire experiment was repeated three different times over a period of nine months, using approximately 40 animals in each series. The basic plan of the experiments was as follows: The animals were x-rayed before hormonal treatment was started. All experimental groups received five daily priming injections of estradiol and were x-rayed again on the 6th day. Additional hormonal treatment was started after this second x-ray and continued for the duration of the experiment. The animals were x-rayed at indicated times thereafter, and a group of untreated animals x-rayed each time served as controls.

EXPERIMENTAL DATA

Due to their nature, the results have been divided for consideration into two groups: the first is a comparison of all groups through the first fifteen days of treatment; and the second shows effects of continued injections of relaxin over a 20 to 25 day period.

1. *Comparison of Experimental Groups*

In each of the series of experiments conducted, the castrated animals used were divided into seven groups as follows:

(A) Control animals which were given no hormonal treatment but which were x-rayed each time the experimental groups were subjected to this stress.
(B_1) Animals treated with estradiol alone.
(B_2) Hysterectomized animals given daily injections of estrogen and progesterone after a five day priming treatment with estradiol alone.
(C_1) Animals treated with relaxin and estradiol after a five day priming treatment with the estrogen alone.
(C_2) Hysterectomized animals given daily injections of progesterone, estradiol, and relaxin after a five day pretreatment with estradiol alone.

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[2] The estrogen used in these experiments was alpha-estradiol supplied by Schering Corporation, Bloomfield, N. J.

[3] The relaxin extracted from sow ovaries was prepared and supplied by Dr. Robert Kroc of the Chilcott Laboratories, Division of the Maltine Company, Morris Plains, N. J.

(D) Animals (non-hysterectomized) given daily injections of estradiol and progesterone after a 5 day priming period with the estrogen alone.

(E) Animals (non-hysterectomized) treated with progesterone, estradiol, and relaxin after the 5 day priming period.

The results of these experiments have been summarized in graphic form in Figure 1. In analyzing these data, it must first be brought out that the

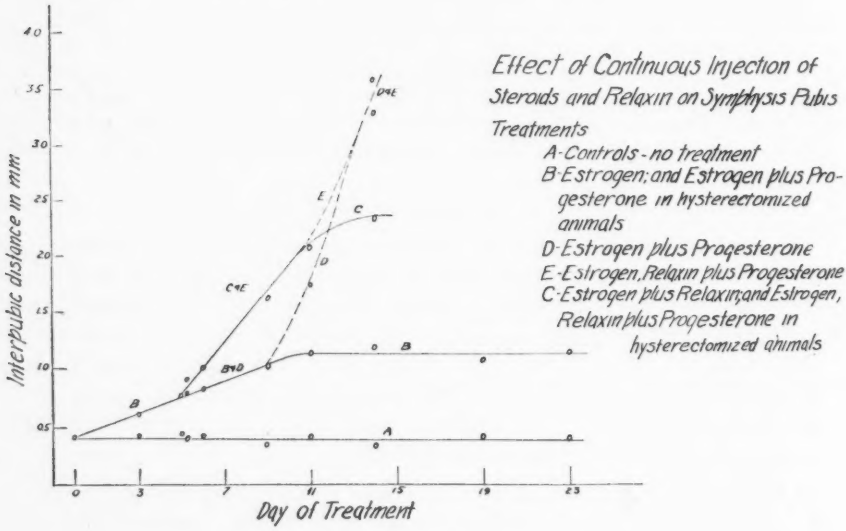

FIG. 1

response of the symphyseal tissue to extracts containing relaxin is exceedingly variable (See Talmage and Hurst, 1950). Therefore, all experimental groups which could not be shown to be statistically different, even though the average value varied considerably, were grouped together. Consequently, the response curves as shown in Figure 1 only approximate the true curve for any one group; but, nevertheless, each group included in a response curve on this graph is statistically different from each group included in the other curves. The quantitative differences are therefore only approximate but are significant.

Certain pertinent conclusions drawn from these results are listed below:

(1) The response to even a low dosage of estradiol (1.0 μg./day) is very marked and appears to be linear for at least the first ten days of treatment (See Talmage, 1950).

(2) Relaxin plus estrogen causes a much greater, although more variable, symphyseal response than does the administration of estrogen alone. This response to relaxin starts to level off after 6 to 10 days of continuous treatment.

(3) The addition of low daily doses of progesterone (1.0 mg./day) to the treatments described in 1) and 2) above shows no effect until 4 to 6 days following the first injection, after which time it causes a symphyseal spread greater than that produced by any other form of experimental treatment.

(4) Hysterectomy nullifies all effects of progesterone. There was some indication that in hysterectomized animals, progesterone might have a slight inhibitory effect on these symphyseal reactions such as has been noted in the mouse (Hall, 1949) but further work is required before this can be definitely established.

2. *Effects of Continued Treatment with Relaxative Substances*

During the course of earlier experiments concerned with the histological changes produced in the symphysis pubis by several steroids and relaxin, it became apparent that the symphyses did not remain relaxed, as determined by the palpation method, throughout the period of relaxin administration. The validity of this observation could be determined best by actual x-ray measurements made at frequent intervals after the initiation of relaxin treatment. Such studies were made, therefore, by administration of relaxin, accompanied by x-rays at 3 to 5 day intervals, over a period of 29 to 25 days. The relaxin used was obtained from three sources: 1) extract of sow ovaries (all further unqualified references to "relaxin" refer to this source); 2) pregnant rabbit serum; and 3) endogenous relaxin produced by injection of progesterone. The symphyseal response of each guinea pig was plotted graphically. Two typical curves are presented in Figure 2, one of an animal treated with relaxin, the other of an animal treated with progesterone.

Study of data obtained from these experiments revealed that, in animals that were also under estrogen treatment, relaxin was unable to maintain a continuous separation of the symphyseal bones. Commencing about the tenth day of relaxin treatment, the symphysis appears to become temporarily and somewhat cyclically refractory to relaxin, contracting and separating at irregular intervals. This phenomenon was observed in all relaxin-treated animals including those in which the dose of the hormone was doubled starting the tenth day of relaxin treatment. The actual time at which this failure of the symphysis to respond first appeared varied from the 8th day of relaxin treatment through the 16th. The degree of contraction undergone by the symphysis varied tremendously; in some

animals the interpubic distance decreased down to the average value obtained for animals given estrogen alone, in others the drop was hardly more than a leveling off process.

That this refractoriness could be attributed to relaxin per se and was not merely a response to an extract of sow ovary was confirmed by a similar

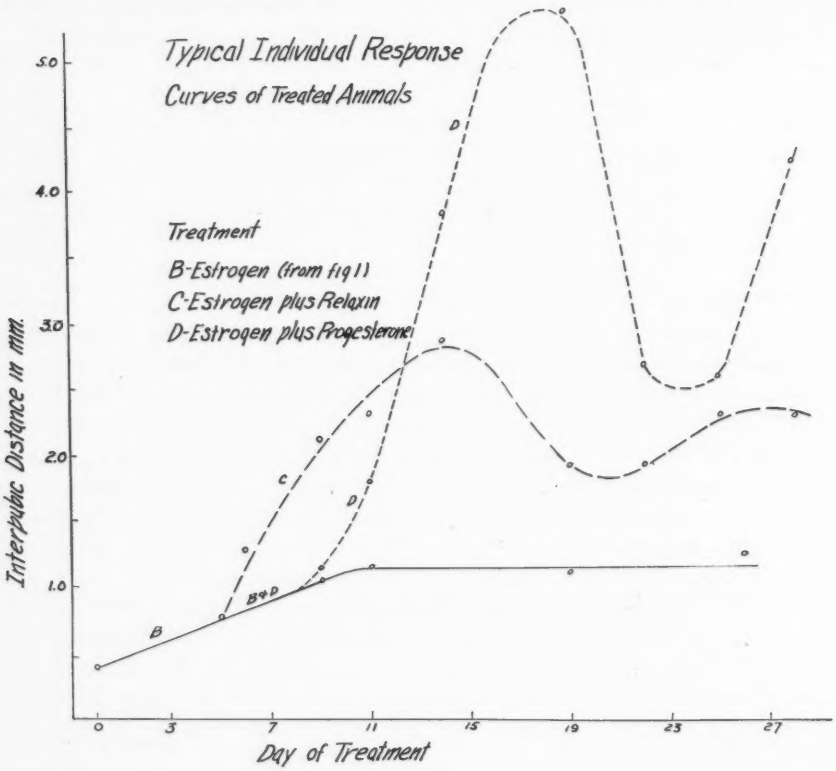

FIG. 2

refractoriness observed in animals treated with progesterone, with pregnant rabbit serum, and with a combination of progesterone and relaxin. Prolonged treatment with pregnant rabbit serum produced this cyclic refractoriness at similar time intervals as with the relaxin obtained from sow ovary. In animals treated with progesterone this phenomenon did not occur until after the 15th day of progesterone treatment and in one of the animals studied, it had not occurred by the 25th day. Considering that there is approximately a five day delay after the first injection of progesterone before sufficient endogenous relaxin is produced to affect the

symphyseal tissue, the equal additional length of time before this refractory phenomenon occurs indicates that the symphyseal tissue responds similarly to endogenous relaxin as to that from exogenous sources. The response pattern of the symphysis of animals given both progesterone and exogenous relaxin indicates that the endogenous relaxin produces an additive effect at the time expected (See Figure 1) but that the later addition of this endogenous material does not postpone the appearance of refractive phenomenon.

DISCUSSION

The work described here has been an attempt to gather more information concerning the process by which the symphysis pubis of the guinea pig responds to estrogen, progesterone, and to relaxin. That the hormones used here all have a qualitatively different effect on symphyseal separation has been confirmed many times by the various investigators listed earlier. These studies substantiate the earlier works in every regard. Certain very interesting, though not entirely explainable, additional observations can be presented here.

The first of these is that endogenous relaxin produced the maximum spread in these experimental animals. This can probably be attributed to the continuous production of relaxin by the uterus versus the sporadic administration of relaxin from external sources. Zarrow and Money (1948) have shown that relaxin disappears very rapidly when injected, indicating that it is almost impossible to maintain a constant level of relaxin in the body.

Separation of the symphysis pubis by estrogen alone has usually been produced with daily injections of estradiol at dosages of 10 μg. or larger, though at the low dosage used in these experiments (1.0 μg. daily) palpable separation has on occasions been noted in animals treated over 25 days. These experiments show that even at this dosage estradiol has a marked effect on the symphyseal tissue, increasing the interpubic distance by a factor of three during the first 10 to 12 days which level appears to be maintained fairly constantly.

The most surprising result was the inability of relaxin to maintain the state of separation of the pubes which had been accomplished through its previous action. The fact that this is true even when endogenous relaxin has caused the separation rules out the possibility of refractoriness to a foreign substance. Corollary studies concerning the effects of relaxin on other physiological endpoints are being carried out to see if this is a specific response of symphyseal tissue or a general body reponse to the homone.

This sensitiveness of the symphyseal tissue to estrogen alone, accompanied by the apparent failure of relaxin to function effectively for a period

of more than 10 to 15 days, would lead one to suspect that in the normal process of symphyseal separation as seen in the pregnant guinea pig, estrogen must be the key hormone, with relaxin acting in a temporary and abettive role. Certainly it must be admitted that the magnitude of separation and the histological condition of the symphyseal tissue as seen in pregnancy has not been approached experimentally by the combined use of estrogen, progesterone and relaxin.

SUMMARY

By use of x-rays, the changes produced in the symphysis pubis of the guinea pig by prolonged treatment with estrogen, progesterone, and relaxin have been studied further. The earlier works describing the differences in the process of symphyseal relaxation as produced by these hormones have been fully substantiated and the following additional results established:

(1) The symphysis pubis is very sensitive even to small amounts of estradiol. Given over a fifteen to twenty day period, 1.0 μg. daily will cause a three-fold increase in the interpubic distance.

(2) The maximum symphyseal spread obtained experimentally resulted from the production of endogenous relaxin by a 10 to 15 day treatment with progesterone (1.0 mg./day).

(3) Relaxin is unable to maintain for more than a short period, the symphyseal spread induced by its previous action. After approximately a ten day treatment with relaxin, the symphysis contracts and expands irregularly even in the presence of continued administration of the hormone.

(4) This refractory phenomenon appears in the symphyseal tissue of animals treated with extracts of the sow ovary, pregnant rabbit serum, and with progesterone.

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THE INFLUENCE OF THE THYROID ON CEREBRAL METABOLISM*

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THE changes in mental activity associated with abnormal thyroid states have usually been explained on the basis of alterations in cerebral metabolism due directly to local excess or deficiency of the thyroid hormone. Such reasoning has received a certain amount of experimental support, if only by analogy. McEachern (1935) has demonstrated a significant increase in the oxygen consumption of liver, kidney, and diaphragm after administration of thyroxin. Jandorf and Williams (1944) using thyrotropic hormone, reported a 50 to 100 per cent increase in oxygen consumption of liver and muscle. (These investigators using adult animals were unable to show any decrease in oxygen consumption in the same tissues following thiouracil administration.) Cohen and Gerard (1937) observed a 30 per cent increase in the cerebral oxygen consumption of animals given thyroxin. In a preliminary study (Fazekas, 1946) we were unable to demonstrate any acceleration of the cerebral oxygen consumption in hyperthyroid rats, nor was there a decrease in the rate of cerebral oxygen utilization as a result of thyroid deficiency. In vivo studies by Himwich and co-workers (Himwich, Daly, Fazekas and Herrlich 1942), done on cretins given thyroxin showed an increase in cerebral oxygen consumption as determined by the arteriovenous technique. The interpretation of the results is difficult since the rate of cerebral blood flow was not quantitatively determined.

Because of the absence of evidence permitting an accurate evaluation of the relative importance of contributing factors found in the intact subject, it seemed wise to approach such an evaluation by first determining the effect of an excess or a deficiency of thyroid hormone on oxygen consumption of the brain in vitro. Further, because of the extreme effect of thyroid deficiency on mentation as observed in human cretinism, it was evident that such studies should encompass the metabolic response of developing

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nervous tissue as well as that of the adult. Experiments were performed on the cerebral cortex of adult and developing rats of various ages. We are not aware of any data on the effect of thyroid hormone in increased or decreased concentrations on the oxygen consumption of the developing central nervous system.

METHOD

Hypothyroidism was induced in newborn rats by placing the pregnant female on propylthiouracil, 0.1 per cent in "Foxchow" ration about two weeks prior to delivery. After delivery the mother was continued on this diet for the duration of the experiment in order to maintain the hypothyroid state in her litter. (It is well known that propylthiouracil is secreted in the mother's milk.) The criteria used to identify rat cretinism were set forth by Hughes (1944). The young animals showed marked physical retardation as evidenced by failure to gain weight and failure of ears, teeth, eyes, shape of head, and tail to develop normally.

A hyperthyroid state was induced in other infantile rats by the subcutaneous injection of 6 gamma of thyroxin daily for the first six days of life. For the next ten days they received 20 gamma daily. Development was precocious in these animals as compared to the normal. Control animals were either untreated litter mates of the hyperthyroid animals or animals of the same age taken from other litters. No differences were observed between animals of the same age regardless of the litter. Adult rats, 100 to 130 days of age, were depleted of thyroid hormone by the administration of 0.1 per cent propylthiouracil in "Foxchow," which was the only food. This was continued for six weeks before sacrifice. Hyperthyroidism was induced in adult rats of the same age by the subcutaneous injection of 100 gamma of thyroxin daily for twenty-five days. The rats were sacrificed at the end of that time. All animals received liberal amounts of brewers' yeast to obviate the possibility of a vitamin B_1 deficiency.

Each animal was sacrificed by decapitation and the brain removed. The various parts of the brain were separated and weighed. If the weight was 150 mg. or less, the total part was used; if it was more, the part was divided into 150 mg. lots by wet weight. As much white matter as possible was removed from the cortex before weighing. The weighed portions were minced and placed in Warburg vessels, each containing 3 cc. of phosphate saline buffer and 0.1 cc. of 10 per cent glucose solution. The oxygen consumption was noted for one hour after equilibration and reported on the basis of 100 mg. of tissue (wet weight).

RESULTS

The results are presented on the accompanying graph. Each point on the curves represents the average of at least six observations. The oxygen consumption of cerebral cortex for the first ten days of life was found to be constant at approximately 75 mm.^{3} 0_2/100 mg. cortex/hour for both the normal and the hypothyroid rat. From 10 to 24 days there was noted in both a gradual and equal increase in the oxygen consumption based on wet weight. After this time the increase showed deceleration, marked at 40 days, so that at 46 days the oxygen consumption had reached adult levels of about 195 mm.^{3} 0_2/100 mg. cortex/hour, and there was no further

change. No significant differences were noted at any time between normal and hypothyroid animals of the same age.

For the first six days of life when hyperthyroidism was being induced, the oxygen consumption of the hyperthyroid rat cortex was the same as that of the normal and hypothyroid animal. The expected increase in oxy-

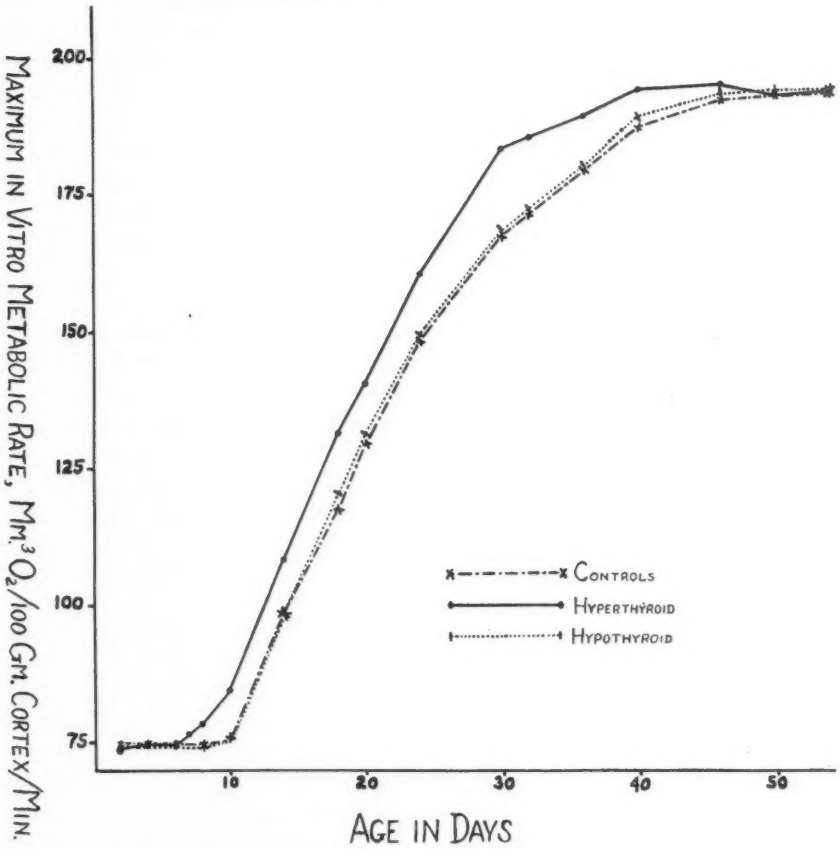

FIG. 1. Cortical oxygen consumption of normal, hyperthyroid and hypothyroid rat brain at various ages. (The vertical ordinate should read "Mm.$^3O_2$/100 *Mg.* Cortex/*Hr.*")

gen consumption appeared on the seventh day instead of after the tenth day as in the controls. The rate of increase was not maximal, however, until the tenth day, so that until the twenty-fourth day (when the increase of controls showed deceleration), the cerebral oxygen consumption of hyperthyroid young rats was equal to that of controls two days older.

Although deceleration of increased cerebral oxygen consumption in hyperthyroid animals did not appear until 30 days of age, so that at this time their cerebral metabolism was equal to that of normal and hypothyroid animals eight days older, this deceleration was more rapid when it did appear. The metabolic rate thus reached the adult level at 40 days (instead of the 46 days required by controls) and did not rise thereafter.

Similar to the finding that the cerebral metabolic rate was essentially the same when normal, hyperthyroid, and hypothyroid rats had reached 46 days of age, the cerebral oxygen uptake showed no significant difference between adult controls and adult animals which had been rendered hyperthyroid or hypothyroid by administration of thyroxin or propylthiouracil.

Dry weights were determined on the tissues, and it was found that the curve of the dry weight oxygen consumption at the various ages in all groups coincided with the wet weight oxygen consumption curves.

DISCUSSION

Our results at first glance seem somewhat surprising in view of the fact that they indicate the metabolic behavior of the brain in the developing hypothyroid animal not to parallel the general physical retardation. Nevertheless, the validity of this observation is anatomically supported by the demonstration (Scow and Simpson, 1945) that rats subjected to complete thyroidectomy at birth do not exhibit in brain and eyeball the marked retardation of development noted in all other tissues. It should be pointed out, however, that the studies which we have reported are concerned only with the developing cerebral cortex. It has previously been shown that the metabolic rate of the cortex reaches adult levels a comparatively long time after that of the lower parts of the brain. It is entirely possible that the retarding influence of hypothyroidism may not be easily detected in a structure the metabolic progress of which already has a relatively flat development curve. Preliminary observations (Fazekas, unpublished data) indicate that the metabolic rate of the midbrain, normally reaching adult levels at approximately 20 days of age, may show a significant retardation due to the hypothyroid state; nevertheless, the adult metabolic level is eventually attained. This is hardly to be considered remarkable since in our studies the adult cerebral metabolic rate was not depressed by induction of hypothyroidism.

The precocious development of hyperthyroid young rats is well established, and it would superficially appear that the more rapid increase toward adult levels of the cerebral metabolism is commensurate with that of the other tissues. However, the true explanation may involve a more fundamental concept. Although in hyperthyroidism the physical development of all tissues does not progress beyond the adult state, with the ex-

ception of that of the brain their oxygen consumption continues to be accelerated. The logical conclusion is that because cerebral metabolism alone apparently is not stimulated by the thyroid hormone above the normal adult level, this level must be equivalent to the maximal metabolic capacity of brain tissue. This hypothesis is supported by the work of Scheinberg (personal communication) and Kety (Sokoloff, Wechsler, Balls and Kety, 1950) whose in vivo studies in man have demonstrated that in hyperthyroidism there is no increase above normal in the cerebral metabolic rate.

Our studies indicate that cerebral oxygen uptake is not directly influenced by increase or even decrease of the thyroid hormone. Furthermore, there is no conclusive evidence to show that any other hormone can directly affect the cerebral metabolic rate. From the teleological standpoint it should not be surprising that in an organism highly dependent on a governing structure for optimal function the metabolism of this structure should be minimally, if at all, influenced by changes in endocrine activity. Nevertheless, it cannot be gainsaid that cerebral function is markedly altered in hyper- or hypothyroidism of developing or adult animals and man. The only explanation for the apparent divergence between clinical and experimental observations is that the former reflects function while the latter records the effects of those processes which maintain the integrity of a structure as well as the effects of its functional activity. In the case of the brain, it is highly probable that the energy requirements for its functional activity are so small that changes are not detectable by present experimental techniques. That the normal oxygen consumption of the developing hypothyroid brain is probably more an index of the metabolic activity supporting the life of the tissue (a process which we should properly describe as the vegetative metabolic activity) than an expression of its functional metabolism finds its parallel in the fact that the cretinoid brain is anatomically normal but functionally abnormal. A similar situation is found in schizophrenia where measurable cerebral metabolism and anatomy are also apparently normal. Finally it is well known that neurotoxic agents invariably cause functional changes before metabolic or anatomical changes are demonstrable in brain tissue.

SUMMARY AND CONCLUSIONS

The metabolism of normal, hyperthyroid, and hypothyroid adult and developing rat cortex was studied in vitro. Cortical oxygen utilization was not influenced by hypothyroidism in developing and adult rats nor did hyperthyroidism appear to influence the metabolism of adult rat cortex. An acceleration toward the normal adult metabolic level was observed in hyperthyroid developing young rats. It is possible that other parts of the

brain show detectable alterations in metabolism in abnormal thyroid states and that there may be quantitatively small changes in functional metabolic activity of the cortex not demonstrable by present experimental methods which are capable of measuring only vegetative metabolism of brain tissue. This portion of the cerebral metabolic activity normally proceeds at the maximum limit of its capacity. The latter concept may help to explain the fact that there has been no conclusive demonstration of acceleration of measurable cerebral oxygen consumption above the normal in vivo level.

ACKNOWLEDGMENT

We are grateful to Dr. Edwin B. Astwood for his interest in and encouragement of this work.

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SOME OBSERVATIONS ON THE PHYSIOLOGIC ACTIVITY OF $\Delta^{4,6}$-DEHYDROCORTISONE (DIENE)

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DURING the partial synthesis of cortisone acetate a small amount of $\Delta^{4,6}$-dehydrocortisone acetate is formed (Mattox and Kendall). This derivative is a diene and in this paper will be designated "diene." It became desirable to determine the physiologic activity of the diene, and to this end experiments were planned which would show the comparable effects of cortisone and the diene on normal and adrenalectomized rats. This paper contains the results of some of these experiments.

Cortisone acetate, when given to experimental animals, has been shown to exert certain well-defined influences on the distribution of some of the cell categories of the peripheral blood and to induce a partial atrophy of the adrenal gland and of the lymphopoietic organs. Three hundred micrograms given daily to adult CBA mice for twenty-one days induced, in this laboratory, a decrease of 54 per cent in the number of lymphocytes per cubic millimeter of blood, an increase of 18 per cent in the number of neutrophilic granulocytes, a decrease of 37 per cent in the average weight of the adrenal glands, and a decrease of 87 per cent in the average weight of the thymus. Comparable changes were induced in a series of adult white rats which were given cortisone at a level of 2.0 mg. daily.

EXPERIMENTAL METHOD

In order to explore some of the physiologic reactions to the diene, $\Delta^{4,6}$-dehydrocortisone, and to contrast them with those induced by cortisone acetate, the following experiment was arranged.* Fifty-five normal and apparently healthy adult male rats

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* The first experiments were carried out with cortisone acetate and with the diene, $\Delta^{4,6}$-dehydrocortisone. The work was then repeated with the acetates of both cortisone and the diene. The results were the same, but it should be pointed out that in the experiments which were carried out for twelve days cortisone acetate and the free diene were used and for all the others the acetates of the steroids were administered.

of the Sprague-Dawley strain were selected from our Institute colony. All were placed on a diet, consisting of purified casein,† 25 per cent; sucrose, 66 per cent; corn oil, 5 per cent; and salt mixture No. 2,† 4 per cent. All vitamin supplements, including vitamins A and D and all essential components of the B-complex, were added to the basal diet in amounts known to be optimal.

Twenty-five of the 55 rats were adrenalectomized. Of these, 5 received 2 mg. of cortisone acetate intramuscularly daily for twenty days, 10 received 2 mg. of the diene daily for twenty days, and 10 remained untreated and served as controls. The adrenalectomized animals were not given salt in their drinking water; their salt intake was restricted to the amount contained in the diet.

Of the 30 normal animals, 5 received 2 mg. of cortisone acetate daily for twelve days, 5 received 2 mg. of the diene daily for twelve days; 5 received 2 mg. of the diene daily for twenty days; 5 received 2 mg. of cortisone acetate and 2 mg. of the diene daily for twelve days. Ten untreated animals served as controls.

All test and control animals were housed individually in wire cages with wire mesh bottoms. Diet was provided in individual feeding cages, and water was constantly available in drinking bottles attached to the cages. All animals were weighed daily, and the amounts of food and water taken by each rat were recorded daily.

RESULTS

A. Food and Water Intake.—There were no significant differences in the amounts of food consumed and water taken daily by the normal rats given cortisone acetate, the diene or the two steroids together. Control rats ate on the average 10.2 gm. per day, cortisone acetate-treated rats ate 10.7 gm. per day, the diene-treated rats ate 12.3 gm. per day, and rats given both cortisone and the diene took an average of 12.2 gm. per day. The volumes of water taken per rat per day varied from 15.3 cc. to 18.2 cc., but significant differences between the groups were not observed.

There were differences noted, however, in the food and water intake of the adrenalectomized animals. The adrenalectomized rats ate well during the first few days after operation, but their food intake had declined to 2.0 gm. per day by the tenth day. Adrenalectomized animals which received cortisone acetate took an average of 11.0 gm. of food per day, while those getting the diene took an average of 13.0 gm. per day during the entire postoperative period. Cortisone acetate-treated and the diene-treated adrenalectomized animals took an average of 18.0 cc. of water daily during the test period, while their untreated controls took 26.0 cc. on the first day, but dropped to 15.0 cc. on the tenth day.

B. Changes in Body Weight.—The average weights of the 5 normal animals given the diene, those given cortisone acetate, and those given the diene together with cortisone acetate, for the twelve-day test period, are shown, in figure 1. Normal animals which received the diene attained far better growth than those which received cortisone acetate alone or

† General Biochemicals Company, Chagrin Falls, Ohio.

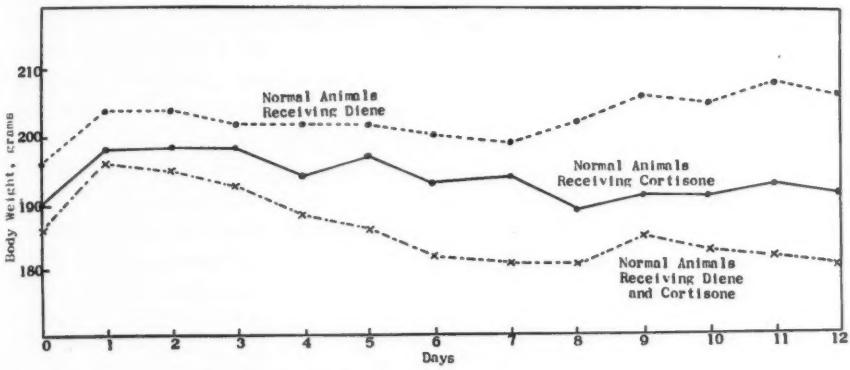

FIG. 1. Weights, during a twelve-day period, of normal animals receiving the diene, receiving cortisone, and those receiving the diene with cortisone.

cortisone acetate combined with the diene: the diene accentuated the effects exerted by cortisone acetate alone. During the longer test period when the diene was given for 20 days (fig. 2) the average gain per rat was 24.0 gm., while during the same period the average gain of the untreated control rats was 62.0 gm. The diene, although less inhibitory to growth than cortisone acetate, did restrict growth to about 40 per cent of that attained by their controls.

The effectiveness of the diene in maintaining the weights of adrenalectomized animals, in contrast with that of cortisone acetate, is shown in figure 3. Under conditions employed in this laboratory adrenalectomized

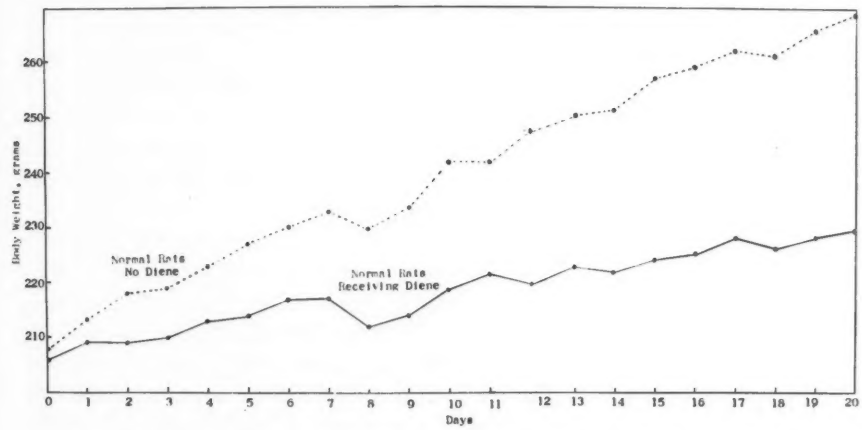

FIG. 2. Veights, during a twenty-day period, of normal animals receiving the diene, and those which did not receive the diene.

rats which are maintained without added salt die within twelve to fourteen days following adrenalectomy. Gain in weight, or survival for longer periods, is indicative of the presence of adrenal rests or other sources of adrenal hormones. In the present test, of the 10 untreated adrenalectomized animals 8 were dead by the eleventh day. Two of which survived the test period were found to have small adrenal rests. The lower curve (fig. 3) portrays the changes of weight of the 8 untreated adrenalectomized animals. All adrenalectomized animals which received cortisone acetate and all which received the diene following operation survived the post-

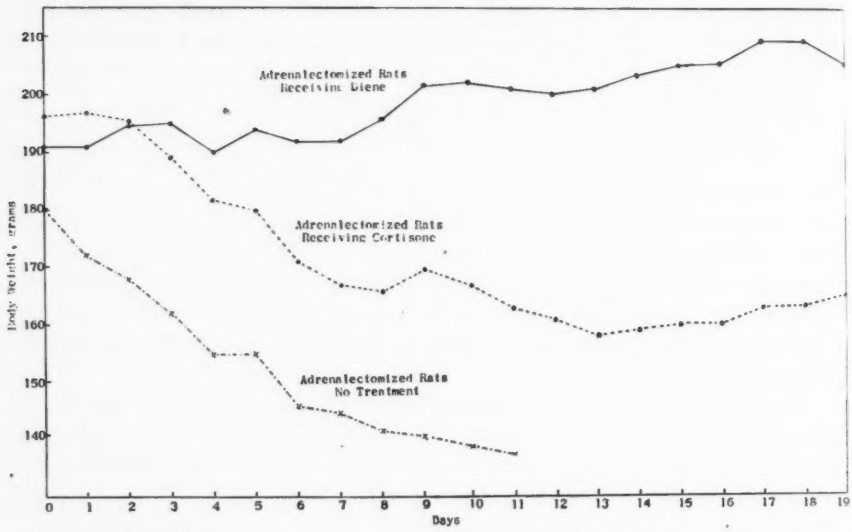

FIG. 3. Weights, during a nineteen-day period, of adrenalectomized animals receiving the diene, receiving cortisone, and those which did not receive either the diene or cortisone.

operative test period (nineteen days). Those which received cortisone acetate, however, lost an average of 32 gm., while those which receive the diene gained an average of 15 gm.

All adrenalectomized animals given cortisone acetate were killed on the nineteenth day. No adrenal tissue was found in any one of the 5 animals. Of the 10 adrenalectomized rats given the diene 5 were killed on the nineteenth day. No adrenal tissue was found in any of these 5. Injections of the diene were discontinued for the remaining 5, and their survival time was determined. One died on the fifteenth day, 2 died on the nineteenth day and the remaining 2 were killed after one month. They had gained weight, and it must be presumed that adrenal rests may have been functioning, although glandular tissue was not identified at necropsy.

C. Changes in the Peripheral Blood.—At the termination of the experiment, the distribution of leukocytes, lymphocytes and neutrophilic granulocytes per cubic millimeter of blood was determined on samples taken from tail veins of all control and test animals. The data so obtained are set forth in figure 4. Cortisone acetate, as was anticipated, induced a marked leukopenia wherein the average leukocyte count was 10,300 cells per cubic millimeter less than that of their controls, whereas, in those animals given the diene the average count was 2,400 cells more per cubic millimeter of blood than the count of their controls. When the diene was given with cortisone acetate the leukopenia was slightly less than when cortisone acetate was given alone; the average count was 7,700 cells less than that of the control.

These changes were caused by a decrease in the number of lymphocytes. The tabulation of neutrophilic granulocytes per cubic millimeter of blood was essentially alike in all four groups, ranging from 2,600 to 3,900 cells. However, the tabulation of the average lymphocyte count showed that with cortisone acetate the number of lymphocytes decreased from a control level of 12,200 to 2,700, while with the diene, in the same amount daily and for the same period of time, the number of lymphocytes increased from 12,200 to 15,600 per cubic millimeter of blood. When the diene was combined with cortisone acetate the lymphopenia was again pronounced, but less so than when cortisone acetate was given alone.

In the adrenalectomized series of animals the effect produced by the diene on the circulating leukocytes was far less than that exerted by cortisone acetate, although a lymphocytolytic effect induced by the diene was obvious. Adrenalectomy resulted in an increase of 45 per cent in the average leukocyte count over that of the normal control group. This increase was caused by the larger number of circulating lymphocytes in the blood stream. Cortisone acetate given to animals without their adrenal glands decreased the number of lymphocytes 70 per cent, an amount which is comparable to that obtained in the animals with intact adrenals.

In the adrenalectomized animals which were given the diene the number of lymphocytes was 42 per cent less than the number of their controls. As stated previously, the diene, when given to intact animals, did not exert a lymphocytolytic effect; but on the contrary there was an increase of 28 per cent in the number of lymphocytes above the control level. These results thus indicate that in intact animals the diene exerted a lymphocytopoietic effect, but in animals without adrenals there was a definite lymphocytolytic influence. This surprising observation was unanticipated and invites further study.

D. Changes in the Weight of Certain Organs.—The average weights of the adrenal glands of the control animals obtained at the end of the experi-

ment, as well as of those which received cortisone acetate, the diene, and both cortisone acetate and the diene, are set forth in figure 4. In the control series, the average weight of the adrenal glands was 19.3 mg. per 100 gm. of body weight. In those given cortisone acetate the average weight was 12.2 mg. per 100 gm. of body weight, a reduction from the control level of 37 per cent. In the animals given the diene a greater atrophy had

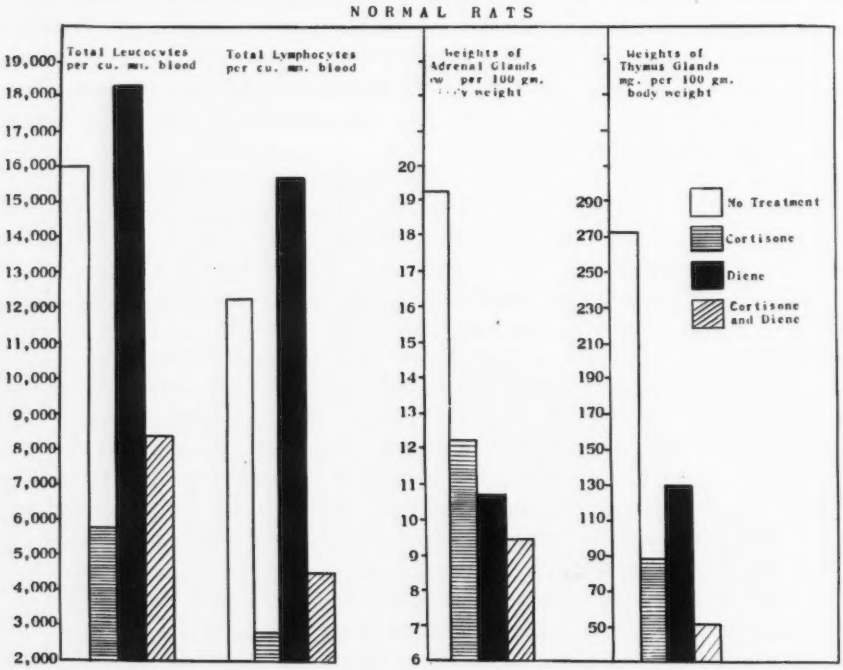
NORMAL RATS
Total Leucocytes per cu. mm. blood
Total Lymphocytes per cu. mm. blood
Weights of Adrenal Glands mg. per 100 gm. body weight
Weights of Thymus Glands mg. per 100 gm. body weight
No Treatment
Cortisone
Diene
Cortisone and Diene

FIG. 4. Total leukocyte and total lymphocyte distributions, the weights of the adrenal glands and thymus of normal control animals, of those receiving cortisone, of those receiving the diene and of those receiving cortisone with the diene.

occurred; the average weight was 10.7 mg. per 100 gm. of body weight, which is a reduction from the control value of 45 per cent. In those animals given equal amounts of both steroids there was a still further atrophy; a corresponding weight of 9.4 mg. was recorded. This is a reduction of 51 per cent from the control values.

Cortisone acetate and the diene exerted an effect on the weight of the thymus (fig. 4). Cortisone acetate induced marked atrophy of this structure. These data, which confirm previous observations, showed that the weight of the thymus per 100 gm. of body weight in the cortisone acetate-

treated animals was 33 per cent of the corresponding weight in the control rats. In the diene-treated animals the average weight was 48 per cent of that of the control group and in those which were given the diene together with cortisone acetate the weight of the thymus was only 18 per cent of the control weight.

Both cortisone acetate and the diene exerted a marked atrophic influence on the weight of the thymus in the adrenalectomized animals. In the absence of the adrenal glands the thymus hypertrophies. In the rats which

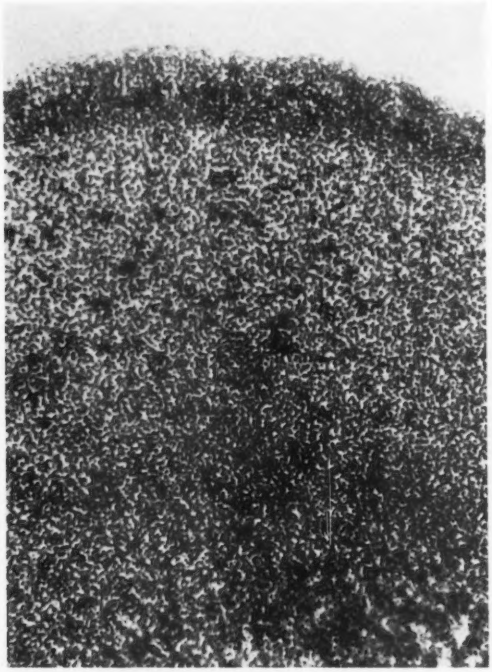

FIG. 5. Frozen section of adrenal cortex of normal animal (fat-soluble stain ×100).

were adrenalectomized there was an increase of 50 per cent in the weight of the thymus. Cortisone acetate prevented this increase and induced an atrophy, so that the weight of the thymus (96 mg.) was but 23 per cent of the attained weight of this structure in the adrenalectomized controls. The diene was again somewhat less destructive than cortisone acetate to the thymic tissue; in these animals the weight of the thymus was 37 per cent of the corresponding weight of the control group.

E. Histologic Changes Observed in the Adrenal and Thyroid Glands.— The adrenal cortex, as is well known, consists of three zones or layers

designated the zona glomerulosa, the zona fasciculata and the zona reticularis, progressing toward the center from the peripheral capsule. When frozen sections of the adrenal cortex are appropriately stained with fat-soluble dyes, the zones containing lipid droplets are clearly delineated. In the control animals maintained on the purified ration the lipid zone in the glomerulosa appeared as a narrow band of cells containing numerous

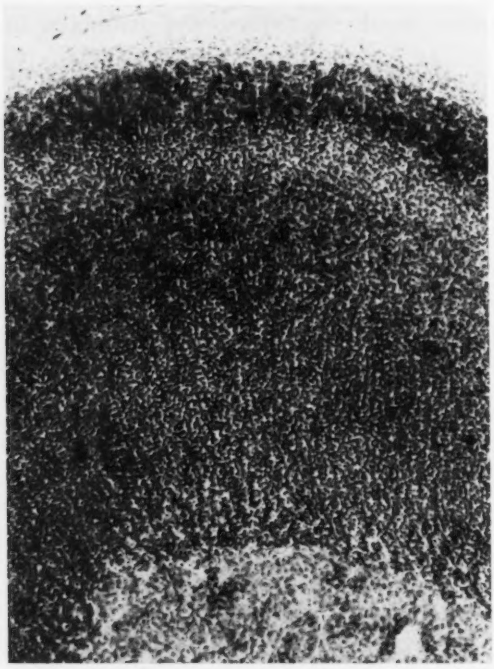

FIG. 6. Adrenal cortex of animal given 2 mg. of cortisone acetate daily for eleven days (fat-soluble stain ×100).

lipid droplets (fig. 5). A lipid-free zone was interposed between the lipid zone of the glomerulosa and that of the fasciculata. The fat-laden area in the latter zone was wide, involving almost all of the fasciculata, even extending into the reticularis.

In the cortices of the adrenal glands of animals which had received cortisone acetate, the lipid zone of the glomerulosa was three times the width of that in the control animals (fig. 6). Although the fat-staining zone in the fasciculata was less extensive in the cortisone acetate-treated animals than in the control animals, the fat droplets were more concentrated in the outer portion of this layer than in the control glands. In the diene-treated animals the zones of both glomerulosa and fasciculata

resembled in width those of the cortisone acetate-treated animals (fig. 7), but the concentration of the fat was somewhat less in the glands of animals given the diene than in those given cortisone acetate. Combining the diene with cortisone acetate intensified the deposition of fat-staining lipids, so that the glomerulosa lipid zone and the fasciculata lipid zone were not only as large as when cortisone acetate was given alone but were far more deeply delineated by the fat stains (fig. 8).

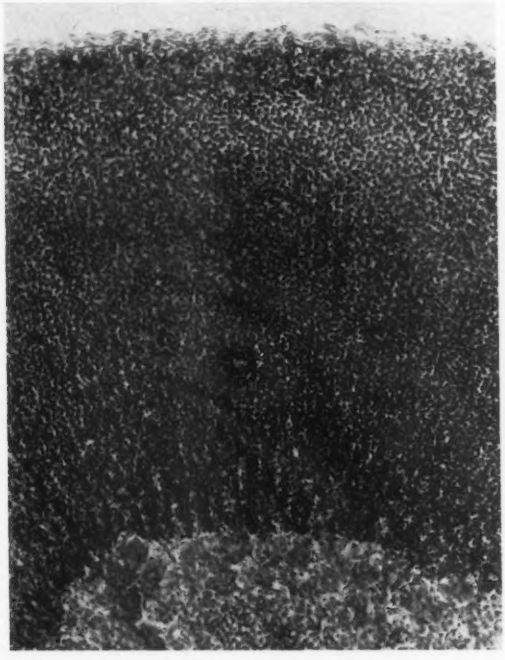

FIG. 7. Adrenal cortex of animal given 2 mg. of the diene daily for eleven days (fat-soluble stain ×100).

Greep and Deane (1949) have reviewed the recent studies on the cytology and cytochemistry of the adrenal cortex. Their data support previous conclusions that the zona glomerulosa regulates fluid and electrolyte balance, while the zona fasciculata secretes the 11-oxygenated-steroids, which are concerned with gluconeogenesis.

Our data show that the lipid zones in the glomerulosa of the cortex in animals given cortisone acetate were greatly enlarged. In animals given the diene alone these zones were far less extensive; but when the diene was given with the cortisone acetate this lipid zone was even more intensely stained, with fat stains, than when cortisone acetate was given alone.

In the fasciculata of the cortex the lipid zone of cortisone acetate-treated animals was more concentrated in the outer layers than in control animals. In diene-treated animals, however, this lipid zone was about as extensive but less concentrated than in those given cortisone acetate; while the combination of those steroids, however, induced a lipid zone, in the

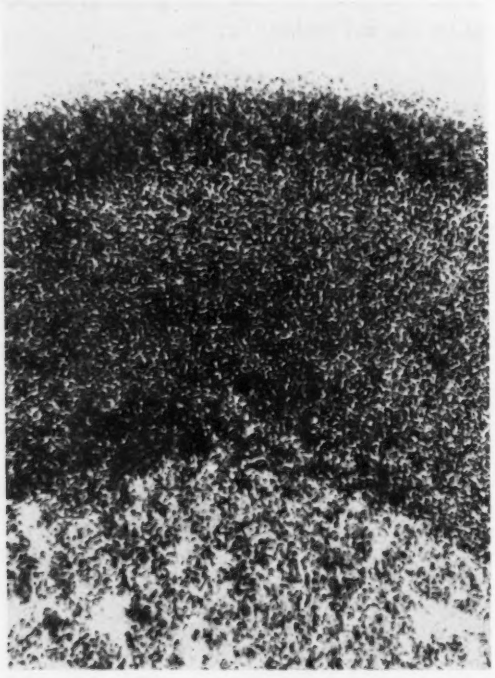

FIG. 8. Adrenal cortex of animal given 2 mg. of cortisone acetate together with 2 mg. of the diene daily for eleven days (fat-soluble stain ×100).

fasciculata, deeply stained and much more heavily laden with fat droplets.

In the thyroid gland of a normal rat the follicular epithelium of the peripheral acini was flat (fig. 9), and each acinus was well filled with colloid. Acini below those at the periphery, closer to the center of the gland, were invariably smaller and were composed of an epithelium more cuboidal in structure. These arrangements were maintained in all normal adult thyroid glands; namely, large peripheral acini with flat or pavement epithelia, and small centrally placed acini with definitely cuboidal epithelia. Cortisone acetate when given in the amounts employed in this study appeared to exert two effects on the thyroid glands of these rats (fig. 10): (1) There was evidence of stimulation of the gland, for the peripheral acini were composed of epithelia which were now cuboidal, and the epithelia of

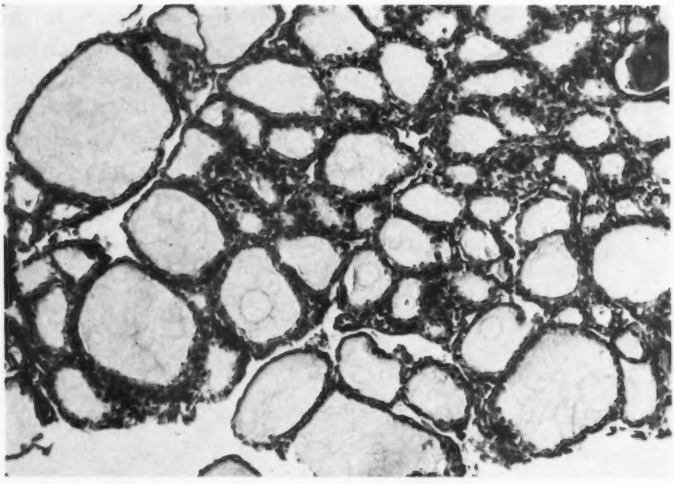

FIG. 9. Thyroid gland of a normal rat (formalin fixation; hematoxylin and eosin ×155).

the central acini were much higher than in control glands. (2) Colloid within the acini had largely disintegrated; the material was granular in appearance and had lost the staining property of normal colloid.

Likewise the administration of the diene in the amounts given induced a stimulation of the thyroid acini (fig. 11) just as cortisone acetate had done, but contrary to the effect of cortisone on the colloid the diene did not exert any effect on the appearance of this material. Normal-appearing

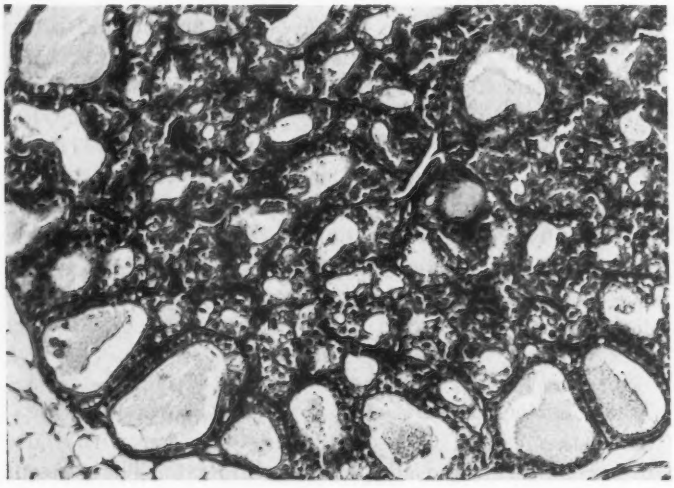

FIG. 10. Thyroid gland of a rat given 2 mg. of cortisone daily for twelve days (×155).

and normal-staining colloid was the rule in thyroid glands of the diene-treated rats, although the epithelia showed very obvious stimulation. When the diene and cortisone acetate were given together (fig. 12), the effect of cortisone acetate on the colloid was evident in most of the acini, but the acinar cell heights were no greater than those in thyroid glands of animals which had received either cortisone acetate or the diene alone.

The acini in the thyroid glands of cortisone acetate-treated rats re-

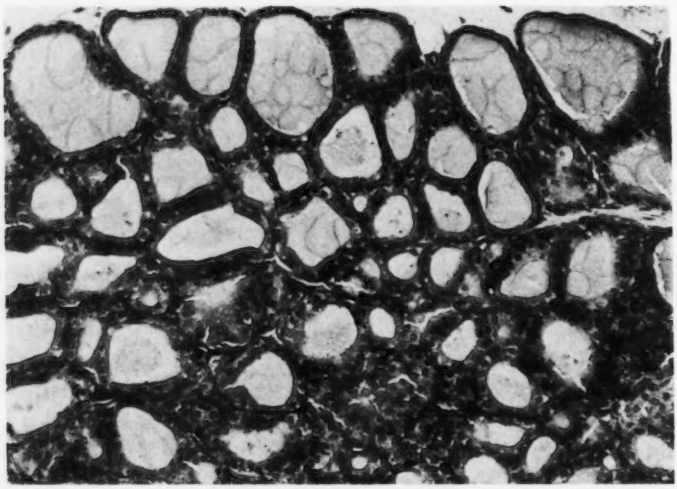

FIG. 11. Thyroid gland of a rat given 2 mg. of the diene daily for twelve days (×155).

sembled those which develop in rats given an antithyroid drug. No attempt will be made to cite the extensive literature describing the response of the thyroid glands to goitrogens (Astwood, 1943 and MacKenzie and MacKenzie, 1943) and we do not have the evidence to conclude that the output of thyroxin was modified by giving cortisone acetate. However, the appearance of the colloid suggests this possibility; and the height of the acinar cells indicates an increased output of the thyrotrophic hormone of the anterior pituitary body. It would be of interest to determine the metabolism of iodine in the glands and the basal metabolic rates of normal and adrenalectomized rats during the administration of cortisone acetate and the diene.

SUMMARY

A study is reported of the results of giving cortisone acetate, a diene, $\Delta^{4,6}$-dehydrocortisone, and cortisone acetate together with the diene to intact and adrenalectomized adult male white rats. These steroids were

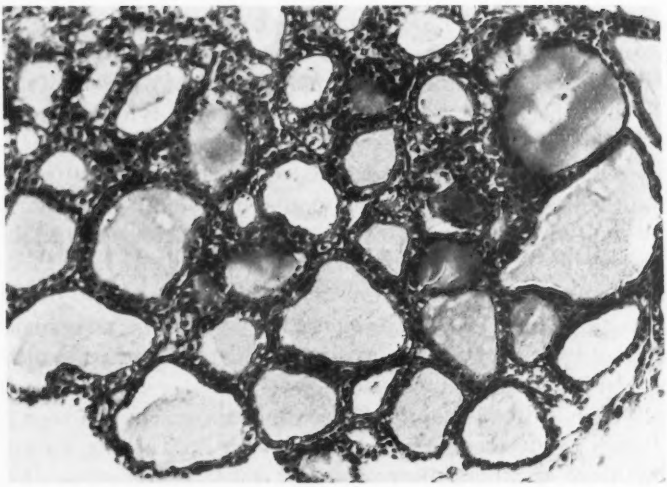

FIG. 12. Thyroid gland of a rat given 2 mg. of the diene and 2 mg. of cortisone daily for twelve days (×155).

given intramuscularly at a level of 2.0 mg. daily for a period of twelve days in one experiment, and for periods of nineteen and twenty days in a second experiment.

Normal animals given these compounds tolerated the diene much more satisfactorily than they did cortisone acetate. Although the intake of food and water by these treated animals was essentially the same as that of the control group, the treated animals gained less weight than that recorded for the controls. In this respect the effect of cortisone acetate was more marked than was that of the diene. When the diene was given with cortisone acetate the loss of body weight was greater than when either compound was given alone.

The diene was much more effective than cortisone acetate in maintaining the body weight of adrenalectomized animals. Under conditions employed in this laboratory adrenalectomized rats rapidly lose weight, and death may occur within ten to fifteen days after operation unless a proper intake of electrolytes is provided. When adrenalectomized rats were given cortisone acetate, but without added electrolytes, their survival time was prolonged, but the body weight declined, so that in the test period of nineteen days the loss of weight was 32 gm. per rat. On the other hand when they were given the diene, the survival time was likewise prolonged, but during the same postoperative period an average gain of weight of 15 gm. was recorded.

In these experiments the lymphocytolytic effect of cortisone acetate was

not observed when normal animals were given the diene. A very real contrast was noted between these two test groups, in that a marked lymphopenia invariably accompanied the administration of cortisone acetate. The diene, on the other hand, appeared to exert a mild lymphocytopoietic influence; the number of lymphocytes per cubic millimeter of blood was actually greater than in the untreated control group. Furthermore the diene induced less atrophy of the lymphoid system, particularly of the thymus, than did cortisone acetate, and the spleen was essentially normal in weight.

Increases of lymphocytes were not observed in the diene-treated adrenalectomized animals. On the contrary lymphocytolysis did occur, but destruction of lymphocytes was less in the diene-treated adrenalectomized animals than in those given cortisone acetate.

Sections of the adrenal glands of these animals, which had been prepared by the frozen tissue technic and stained with lipid stains showed differences which are of much interest and warrant further study. Fat stains applied to the adrenal cortices of normal animals show a narrow lipid zone in the glomerulosa, and a much wider zone in the fasciculata. Cortisone acetate increased the width of the lipid zone in the glomerulosa and intensified the lipid zone in the peripheral layer of the fasciculata. These effects were less marked after administration of the diene, but when the diene was combined with cortisone acetate, the lipid zones of both the glomerulosa and the fasciculata were greatly intensified.

Sections of the thyroid glands of these four groups of animals show that administrations of either of these steroids modified the morphologic appearance of the gland. Cortisone acetate alone, the diene alone, or cortisone acetate given with the diene induced increases in the height of the acinar epithelia. Measurements were not made but the degrees of stimulation appeared to be much alike in the thyroids of all three groups of animals studied.

There was a marked difference between cortisone acetate and the diene in respect to the appearance of the colloid within the acini. The diene did not modify the normal appearance of the colloid. Cortisone acetate induced a granular appearance of the colloid, a loss of the normal staining property and disintegration of the material.

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ANTITHYROID ACTIVITY OF 5-HALOGENO-2-THIOURACILS*

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IN A previous paper (Barrett, Goodman, Dittmer, 1948), the synthesis of 5-halogeno-2-thiouracil and of 6-methyl-5-iodo-2-thiouracil was described. Three derivatives were found to possess antithyroid activity as measured by their ability to increase thyroid weight in the rat and to decrease thyroid iodine. A fuller investigation has now been made, and we wish here to give a more detailed account of their antithyroid behavior.

One of the purposes we hoped to achieve in the synthesis of these compounds was that of providing an antithyroid drug which might render unnecessary the simultaneous use of iodine and thiouracil as practiced preoperatively in patients with hyperthyroidism, and which might alleviate certain undesirable clinical defects in thiouracil or in its 6-alkyl derivatives (Christian, 1946). Interest naturally centered around the 5-iodo derivative, as it would be composed of a presumed antithyroid moiety plus an essential thyroid metabolite.

EXPERIMENTAL

A preliminary oral test in rats with 5-bromo-2-thiouracil disclosed that this type of derivative, while less active than 2-thiouracil, still possessed appreciable antithyroid activity. For a fuller testing of the three halogenated derivatives (Cl, Br, I), we obtained a colony of 140 immature Wistar rats, weighing approximately 75 gm. each, and divided them into 7 groups with 10 of each sex per group. Both 2-thiouracil and 6-methyl-2-thiouracil were used as antithyroid standards, and also a mixture of thiouracil and potassium iodide for further comparison of the effect of the iodinated drug. Groups on these latter treatments received the same amount of iodine per day. In order to avoid the unknown factors of gastrointestinal absorption and destruction and thus secure better comparisons of activity, the drugs were ground to a fine powder, suspended in sesame oil, and injected subcutaneously in the amount of 16 micromols per day per 100

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gms. body weight. The total injection period was 15 days. In order to determine any relation between length of treatment and degree of response, five animals from each group were sacrificed at the end of each five days of the injection period. At the end of 15 days the remaining animals were allowed a 5 day recovery before sacrifice to determine any after-effect. At the time of killing the animals and freshly removed thyroids, adrenals, and pituitaries were weighed. The right lobe of all thyroids was used for iodine analysis, the left lobe for histological examination.

RESULTS

The antithyroid action of the various compounds is summarized in Table 1. Thyroid weights are recorded as mg.%, and thyroid iodine as μg.% based on the weight of the fresh gland. Also, for easy comparison of effect, the experimental values are recorded as per cent change from the

TABLE 1. EFFECT OF INJECTED 5-HALOGENO-2-THIOURACIL ON THE RAT THYROID

Treatment	Days treated:	5				10				15				Recovery			
	Thyroid: Weight			I_2		Weight		I_2		Weight		I_2		Weight		I_2	
		Mg.	% Diff.	Mcg.	% Diff.	Mg.	% Diff.	Mcg.	% Diff.	Mg.	% Diff.	Mcg.	% Diff.	Mg.	% Diff.	Mcg.	% Diff.
1. Control: sesame oil		10.3	0	73	0	9	0	62	0	8.7	0	58	0	9.8	0	54	0
2. Thiouracil		14.2	+42	18	−68	13.6	+51	11	−83	13.1	+50	7	−88	10.6	+8	27	−50
3. Thiouracil+KI		14.9	+45	24	−60	13.0	+44	8	−87	14.0	+61	7	−88	11.4	+19	18	−60
4. 5-Chloro-2-thiouracil		12.1	+21	37	−46	9.6	+7	31	−50	13.9	+60	17	−70	10.2	+4	23	−58
5. 5-Bromo-2-thiouracil		10.6	+2	39	−44	11.7	+30	34	−48	9.5	+10	23	−60	10.8	+10	28	−48
6. 5-Iodo-2-thiouracil		11.3	+10	40	−43	11.5	+28	33	−49	10.4	+19	25	−55	8.1	−17	32	−40
7. 6-Methyl-2-thiouracil		12.0	+20	16	−78	17.1	+90	12	−81	15.0	+73	6	−90	11.8	+20	18	−60

control values. Aside from the changes described for the thyroids, no other significant changes were found.

Thyroid weight changes produced by the halogenated thiouracils were so irregular that any comparisons based on this change are difficult and uncertain. It may be said only that, compared to thiouracil, production of thyroid hyperplasia by these derivatives is slower, and, with the possible exception of the chloro derivative, is much less extreme. Since each value in the table represents an average of only five glands, the decrease in thyroid weight found between the 10 and 15 day periods for groups 5 and 6 may indeed be fortuitous. However, Mackenzie and Mackenzie (1943) have also reported a diminution in weight of the rat thyroid occurring after 19 days of antithyroid treatment.

In contrast to the thyroid weight changes, the decrease in thyroid iodine for all the treated groups was rapid and fairly uniform with time. In agreement with earlier results by Astwood and Bissell (1944) we found a marked iodine loss in as short a time as five days, and concluded that this change provides the better basis for determining antithyroid potency. On this basis, the halogenated derivatives were judged to be about a third less

active than thiouracil and, among themselves, to be of about the same order of potency. In common with other workers, we did not succeed in altering the effect of thiouracil on thyroid weight and iodine content by simultaneous administration of an equivalent amount of potassium iodide.

The brief recovery period indicates only the relative rates at which the thyroids of treated animals returned to normal. In this experiment we found no sex differences in response to the antithyroid treatment, and none of the animals displayed toxicity.

In view of the differences found between the action of 5-iodo-thiouracil and that of the thiouracil-potassium iodide mixture, these compounds were investigated further by oral administration to rats. This fourteen-day study is summarized in Table 2. The amount of iodinated drug used here

TABLE 2. EFFECT OF ORAL 5-IODO-2-THIOURACIL ON THE RAT THYROID

Treatment	Thyroid		I_2		Degree goiter
	Mg. %	% Diff.	Mcg. %	% Diff.	
1. Control	8	0	61	0	0–+1½
2. Thiouracil	16.3	+104	9.2	−85	+3–+4
3. Thiouracil+KI	13.6	+ 70	16	−74	+3
4. 5-Iodo-2-thiouracil	10.4	+ 30	32	−48	+1½

was increased to one and a half times the amount of thiouracil used in an attempt to obtain the same effect by both compounds. The potassium iodide in the mixture was increased to a like amount. It will be noted that while the extra iodide resulted in some amelioration of the action of thiouracil, the larger amount of iodothiouracil did not cause a correspondingly larger effect on the thyroid. Also, as indicated by the degree of goiter produced, it was not possible to distinguish histologically between the thyroid of a control animal and that of an animal treated with 5-iodothiouracil.

The histological appearance of thyroid glands from these experiments is shown in the accompanying Plate. The thyroid lobes reserved for histological examination were cut at 5 micra, and stained by a special method (Wilgus, Gassner, Patton, Gustavson, 1941) based on Mallory's triple connective tissue stain. Sections of representative glands shown in the Plate demonstrate the typical histological changes produced by the compounds used in this experiment. We wish to call attention especially to the goitrogenic changes produced by the thiouracil-potassium iodide mixture as compared to the normal-appearing glands produced by treatment with 5-iodo-2-thiouracil. (Figs. 3 and 6, Plate I).

DISCUSSION

The results reported here from the use of thiouracil or of the 6-methyl analogue represent no new findings. The same is true of the simultaneous

use of thiouracil and potassium iodide, since it is generally agreed that neither inorganic iodide nor diiodotyrosine prevents the goitrogenic action of thiouracil unless given in very large amounts. Ander *et al.* (1945), who synthesized and tested a number of thiouracil derivatives, found that polar groups of various kinds, when substituted into the 5 or 6 position of thiouracil, largely destroyed antithyroid activity. Hence, it was interesting in these experiments to find that the 5-halogeno derivatives of thiouracil possessed appreciable antithyroid activity despite the polar nature of the halogens. In the absence of evidence either way, it is possible that the activity of these derivatives is contingent upon their dehalogenation in the body. However, as the results obtained with the thiouracil-potassium iodide mixture differed in all respects from those obtained with 5-iodo-thiouracil, deiodination of this compound to produce thiouracil plus an iodide would not explain its unique effect—i.e., a small increase in thyroid weight, a marked decrease in thyroid iodine, and an essentially normal histology. If the deiodination of the compound took place directly in the thyroid, then it would be expected that the liberated iodine would repress the goitrogenic response to the thiouracil moiety. Again, if this compound were deiodinated to produce a thyroxine-like substance, or a substance capable of supplying utilizable iodine to the thyroid, the goitrogenic response would again be repressed. At present it is impossible to choose any of these various possibilities, but it is pertinent to point out that Dvoskin (1947) has shown that different forms of iodine can have quite

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FIG. 1. Thyroid of Rat I-5 of the untreated controls which appears normal after 58 days. (Grade 0) ×300.

FIG. 2. Thyroid from Rat II-11 treated with 5 mg. thiouracil daily for 27 days, followed by 10 mg. thiouracil for 22 days. There is severe hyperplasia and hypertrophy of cells with masking of follicular structure and absence of colloid. (Grade +5) ×300.

FIG. 3. Thyroid from Rat III-21 which received thiouracil as noted above and iodine as potassium iodide equimolar to the iodine present in the iodinated compounds. Some reduction of goiter is evident. While follicular structure is discernible, little colloid is present and hyperplasia is still pronounced. (Grade +3.5) ×300.

FIG. 4. Thyroid from Rat IV-25 of the group treated with potassium iodide for the same period of time and at the same level as group III. Minor involutionary changes are seen as well as some hyperplasia, with some disturbance of the normal relationship of cells and colloid to follicular structure. (Grade +1) ×300.

FIG. 5. Thyroid from Rat V-49 which received 5-iodo-2-thiouracil corresponding to equimolar amounts of thiouracil and iodine as potassium iodide fed in Group III. At the end of 58 days of treatment the gland shows a mixture of active and inactive follicles with hyperplastic and atrophic epithelia. (Grade −1 to +1.5) ×300.

FIG. 6. Thyroid from Rat V-43 of the same group following 58 days of treatment and 17 days after drug withdrawal. Note the inactive follicles with extremely low epithelium and increased fibrous tissue. (Grade −2) ×300.

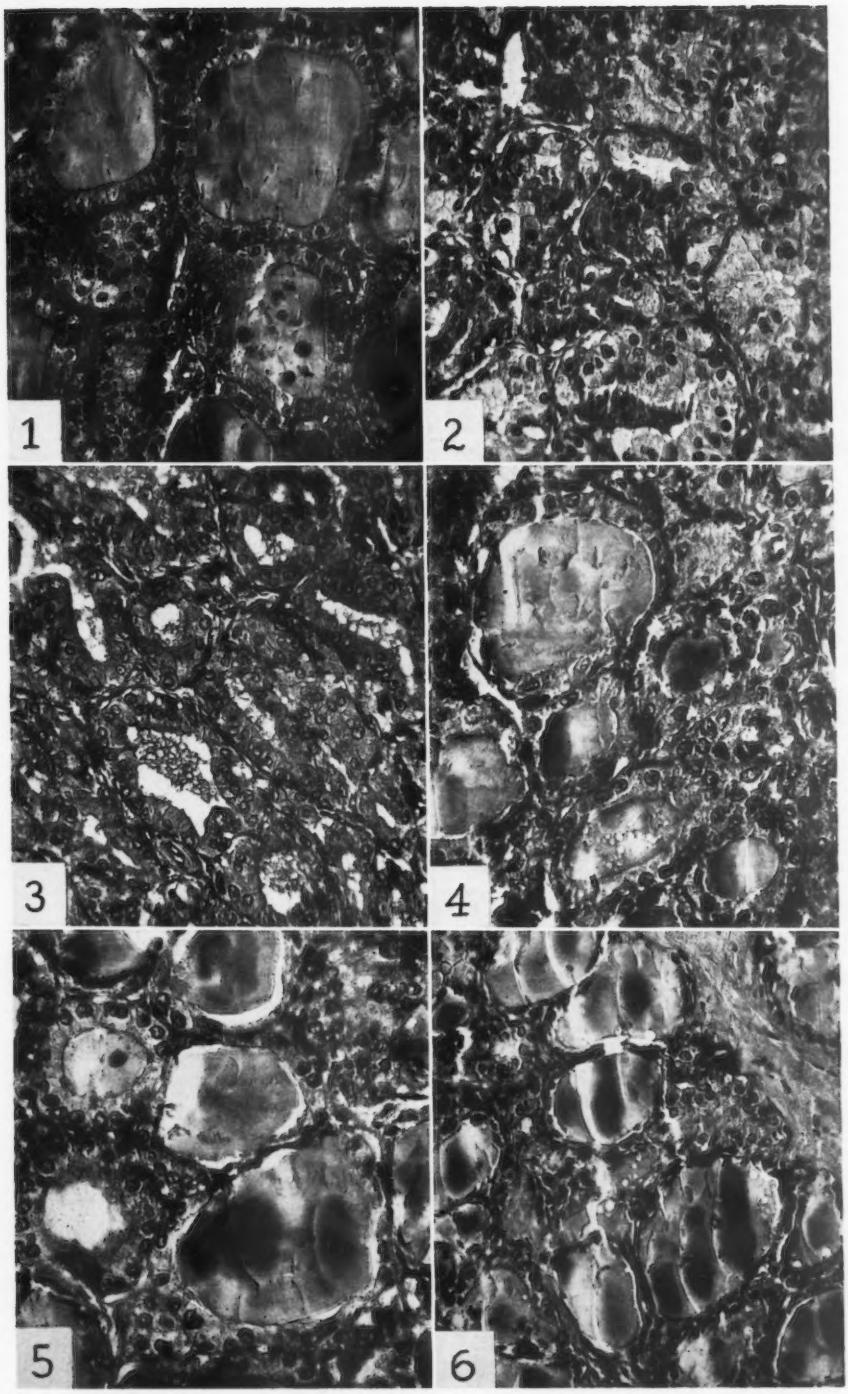

PLATE I

different effects on thyroid histology. Whether the results obtained here with the iodinated thiouracil could be reproduced by a mixture of thiouracil and a particular organic iodide (other than thyroxine) could be determined only by performing the particular experiment.

To mention again one of the original objects in this work, it is apparent that if the effects of 5-iodo-2-thiouracil on the thyroid were accompanied by a decreased BMR, the drug would have obvious clinical attraction for the treatment of human hyperthyroidism, since the chief defects of thiouracil itself would be avoided—production of goitrous glands accompanied by hyperemia and friability. An experiment was subsequently carried out to test this possibility, and the results are described in a separate paper.

SUMMARY

The 5-halogeno-2-thiouracils possess antithyroid activity in the rat, whether administered orally or by subcutaneous injection, as measured by their effect on thyroid weight, iodine content, and histology. Their effect is somewhat weaker than that of 2-thiouracil itself. The most interesting result was that obtained with the iodo derivative which caused (1) a small increase in thyroid weight, (2) a marked decrease in thyroid iodine and, (3) no essential change in the histology of the thyroid. Similar effects were not produced by equimolar amounts of thiouracil and potassium iodide nor by increasing the potassium iodide to 1.5 equivalents.

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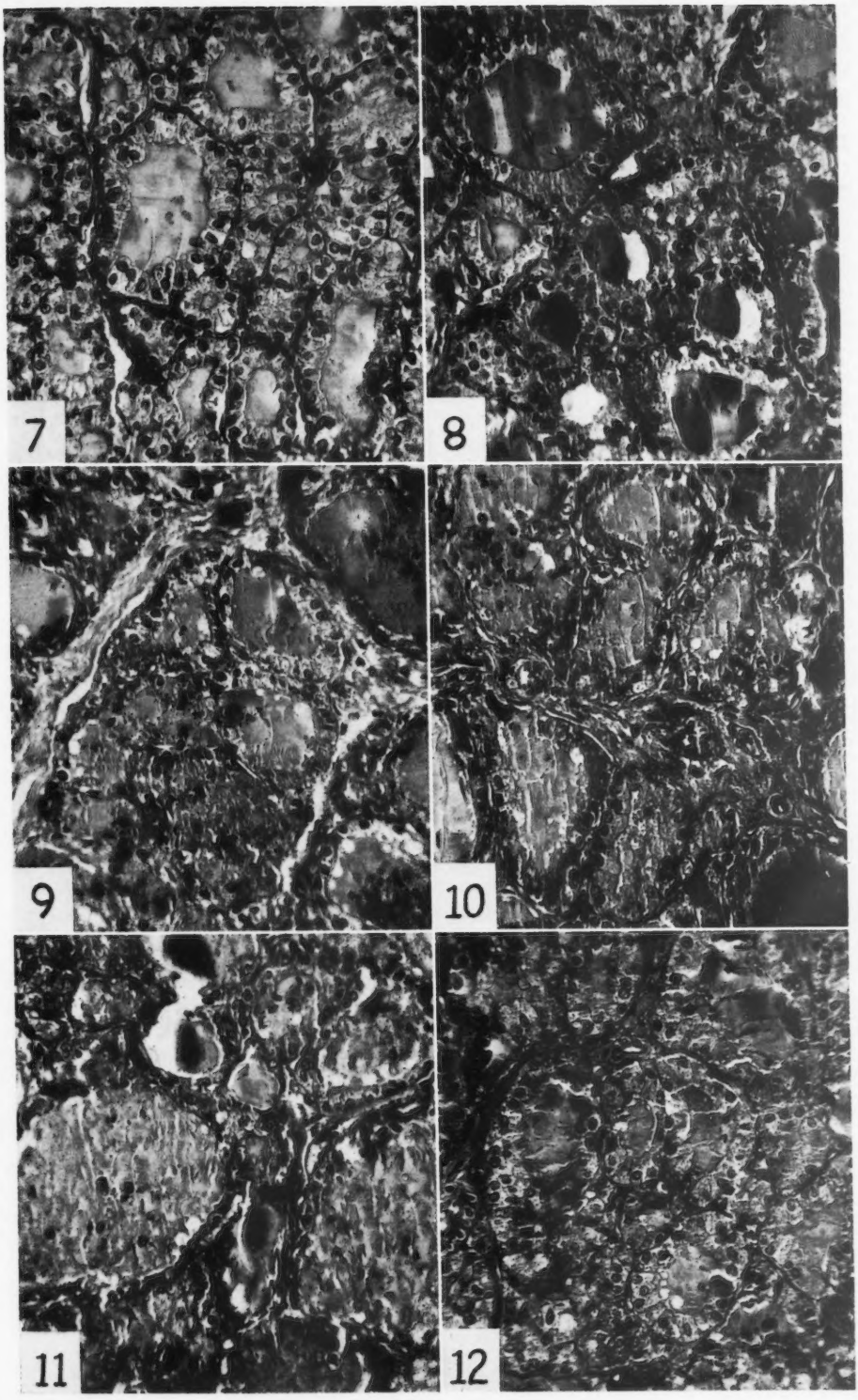
FIG. 7. Thyroid of Rat VI-57 after 37 days of treatment with 5-bromo-2-thiouracil showing a rather uniform hyperplasia and hypertrophy of the follicular cells. (Grade +3.5) ×300.

FIG. 8. Thyroid of Rat VII-62 treated for 19 days with 5-chloro-2-thiouracil. The goitrogenic effect of thiouracil is shown but to a lesser degree than that of the unsubstituted thiouracil. (Grade +2.8) ×300.

FIG. 9. Same as Fig. 8 showing another area of the gland with considerable atrophy, necrosis, and some fibrosis. This mixture of active and inactive follicles was similar to that in rats treated with 5-iodo-2-thiouracil. (Grade −2) ×300.

FIG. 10. Thyroid of Rat VIII-70 which received 5-iodo-6-methyl-2-thiouracil for 58 days. Again the gland shows a mixture of goiter and degeneration. (Grade −2 to +2.5) ×300.

FIGS. 11–12. Thyroid of Rat VIII-67 of the same group showing the great variation in histological appearance noted 17 days after 5-iodo-6-methyl-2-thiouracil was withdrawn. Many follicles appeared atrophic with degenerated epithelium (Fig. 11) with a grading of −2.5 while other areas were definitely goitrous. (Grade +3.5) ×300.



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EFFECT OF 5-HALOGENO-2-THIOURACIL AND OF 6-METHYL-5-IODO-2-THIOURACIL ON OXYGEN CONSUMPTION IN RATS*

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IN the preceding paper it was shown that the 5-halogeno-2-thiouracils are active antithyroid compounds in the rat as measured by their ability to lower thyroid iodine and to increase thyroid weight whether the compounds are given orally or subcutaneously. Since neither of these thyroid changes is necessarily accompanied by a decrease in BMR, the object of this work was to determine whether these halogen derivatives could also lower the BMR of the rat. The oxygen consumption (OC) of the treated animals under standard conditions was chosen as a criterion of anti-metabolic potency.†

EXPERIMENTAL

A colony of Sprague Dawley male rats, approximately 80 days old, was divided into 9 groups for treatment as follows: 1. Control; 2. Thiouracil; 3. Thiouracil plus potassium iodide; 4. Potassium iodide; 5. 5-iodo-2-thiouracil; 6. 5-bromo-2-thiouracil; 7. 5-chloro-2-thiouracil (five animals); 8. 6-methyl-5-iodo-2-thiouracil; 9. Thyroidectomized (five animals). The control group contained 15 animals; others, except as indicated, contained 10. All compounds were administered in the drinking water at a level of 39 umols per day per 100 gms. body weight (39 umols = 5 mg. thiouracil). Later it was necessary to double this dosage to maintain an initial response. The drugs were administered for a total of 58 days except for the bromo and the chloro derivatives. Because of difficulties of synthesis, the quantity of chloro derivative was sufficient for five animals for only 31 days, while animals receiving the bromo derivative began to display toxicity after a month; this group was discontinued after 38 days. Group 9 was thyroidectomized on the 15th day of the experiment after it had been ascertained that their OC was in the same range as that of the controls. Drug administration was discontinued after 58 days, but OC measurements were continued until all values returned to the control level. Measurements were made daily on 3 to 5 animals from the control group and on a like number of animals from one or two experimental groups which had been treated for the

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† Since completion of this work, a paper by R. H. Williams and coworkers (1949) has appeared in which 5-iodo-2-thiouracil is shown to be an effective antithyroid compound for the treatment of human hyperthyroidism.

same length of time. These animals were previously starved for 24 hours while housed at 27–28° C, and all OC measurements were made at the same temperature.

The apparatus for the OC measurements was essentially like that devised and used by Moses (1947) for the same purpose except for the substitution of a calibrated 20 ml. oxygen bulb in place of the Krogh-type respirometer which was not available for this work. The low-speed kymograph was connected to the system by a rubber tambour, and the kymograph curve obtained proved to be quite sensitive to small movements of the animal. Three consecutive runs were made on each animal, and the time required to consume the 20 ml. of oxygen was measured with a stop watch. Measurements were considered acceptable when the kymograph curve indicated no movement of the animal, and when the range of the three measurements did not vary more than 10 seconds. However, the variation was generally less than 10 seconds in a total consumption time usually ranging from 2 to 4 minutes.

At intervals two animals from each of several groups were killed to determine any concurrent thyroid changes. All thyroids were examined as described in the preceding paper, and trachea of the thyroidectomized animals were serially sectioned and examined. One animal had regenerated a small amount of thyroid tissue; values for this animal were discarded.

RESULTS

The results of the OC measurements are shown in Fig. 1. Values for the untreated control animals were taken as a zero base line, and values for

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FIG. 1. Thyroid of Rat 1-16-F of the untreated controls. The effects of relatively low iodine (grade +1) are shown by the increased cell height and granulated cytoplasm. Dense colloid is present. ×300.

FIG. 2. Thyroid from Rat 2-11-M receiving thiouracil showing the severe type of goiter (grade +4) produced by this compound. Hyperplasia and hypertrophy of the follicular cells are evident as is the absence of colloid and the heavy granulation of the follicular cytoplasm. ×300.

Fig. 3. Thyroid from Rat 3-9-F receiving thiouracil and KI. While there is some hypoplasia the gland is still severely goitrous (grade +4). Note the increased vascularity. ×300.

FIG. 4. Thyroid from Rat 4-10-F receiving 5-chloro-2-thiouracil. The goitrous picture is somewhat milder (grade +3). The follicles are generally smaller and some of them contain colloid. Hyperplasia, however, is still severe. ×300.

FIG. 5. Thyroid from Rat 5-5-F receiving 5-bromo-2-thiouracil. A mild goiter (grade +2) is shown as indicated by the increased number of cells, increased cell height, and smaller follicles. ×300.

FIG. 6. Thyroid gland from Rat 6-4-F receiving 5-iodo-2-thiouracil. This gland exhibits little goitrogenic effect (grade +1). Compare with Fig. 3 which received equimolar amounts of thiouracil and iodine as KI. ×300.

FIG. 7. Thyroid gland from Rat 7-7-F receiving 6-methyl-2-thiouracil. This group showed the most severe goiter encountered in the experiment, graded at +5. Note the extreme hyperplasia of the follicular epithelium, the distortion and obliteration of follicular structure, as well as the absence of colloid. The cells are highly granular and show a greatly increased height. ×300.

the treated animals were plotted as a per cent difference from the control. Abbreviations as T (thiouracil), 5-Cl (5-chloro-2-thiouracil) etc., indicate the treatment of the different groups. All oxygen measurements were corrected for temperature and pressure, then calculated as ml. oxygen per

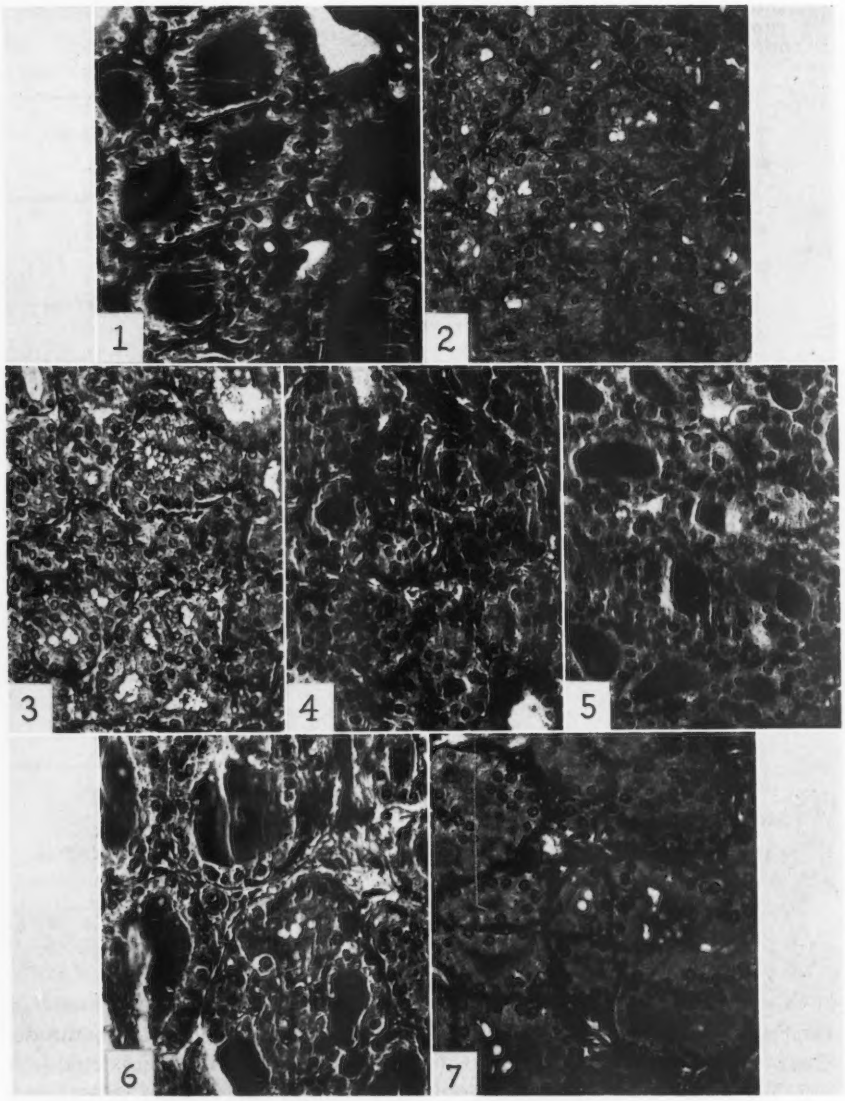
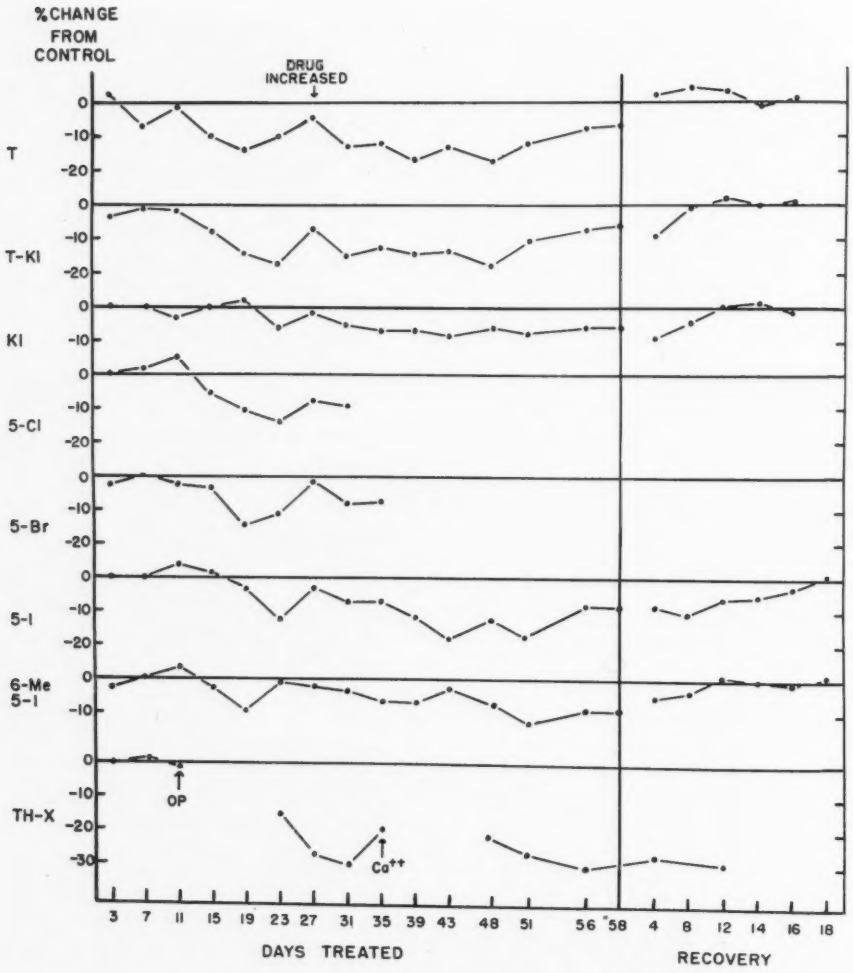


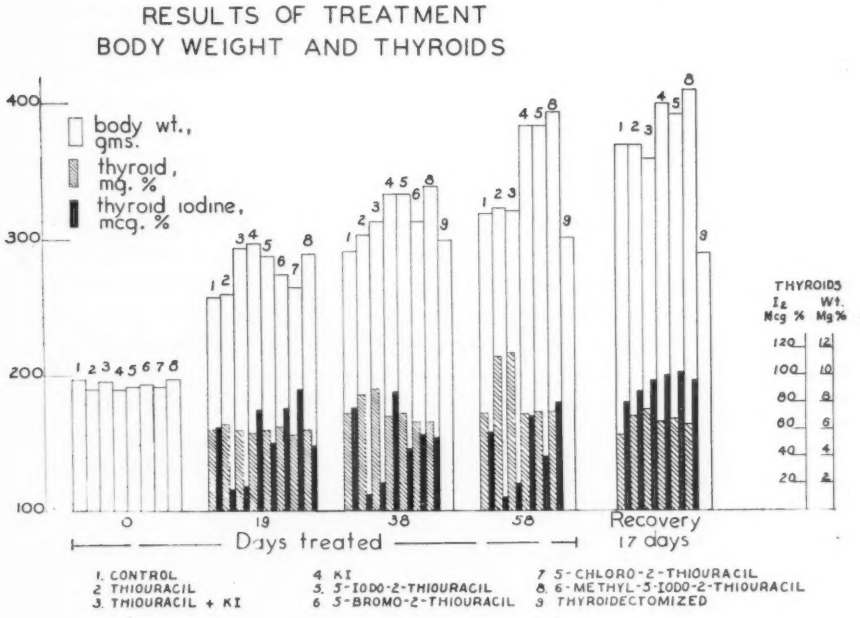
PLATE I

minute per 100 gm. body weight. On this basis, actual values for the control animals varied between 1.6 and 1.72 ml. of oxygen throughout the experiment.



Of the halogenated thiouracils, the 5-iodo derivative was slowest in causing a decreased OC, but it eventually produced about the same decrease as did thiouracil or the thiouracil-potassium iodide mixture (approximately 18%). The chloro and the bromo derivatives acted very similarly to thiouracil so far as it was possible to examine them. On the

basis of these measurements none of the five treatments could be distinguished. A most unexpected finding was the greater antithyroid potency of 5-iodothiouracil compared to the 6-methyl analogue, since the reverse holds with the uniodinated compounds. None of the drugs produced as much metabolic depression as did thyroidectomy. Starting on the 35th day, this group was given a 1% solution of calcium gluconate as drinking water in order to alleviate body tremors noticeable two to three weeks after the operation. Their OC finally became stabilized at 25–30% below the control level.



Measurements made during the recovery period revealed that previous treatment with some form of iodide prolonged the time required by the animal for returning the OC to normal. This "after effect" was rather pronounced following treatment with 5-iodothiouracil, while no such effect was evident with thiouracil.

Results of treatment on body weight and on thyroid weight and iodine content are shown in Fig. 2. The rather striking weight gains made by groups 4, 5, and 8 in this experiment were due to a greater overall body development rather than to mere deposition of fat. If this result was related to the extra dietary iodine given these groups, it is noteworthy

that the group receiving the thiouracil-potassium iodide mixture did not show the same result. Group 6, receiving 5-bromothiouracil, gained weight satisfactorily during the first month of treatment but failed to gain any weight during the following week. Hence, this compound is considered to be somewhat toxic and suitable only for short-term studies.

Treatment with thiouracil and with thiouracil-potassium iodide produced the usual depletion of thyroid iodine and depressed the metabolism of the animals after 19 days of treatment but, surprisingly, produced no change in thyroid weight in the two animals tested at this time. A maximum thyroid weight increase of 43% was produced. After 58 days, none of the halogenated derivatives produced any thyroid weight increase. While there is no obvious explanation for the resistance to thyroid hyperplasia displayed by this particular colony, it again indicates that thyroid weight change is not a sensitive or reliable criterion for judging antithyroid —or antimetabolic—potency. The increased thyroid iodine shown after 19 days of treatment with the chloro and the bromo derivative is a questionable result, but unfortunately these compounds could not be continued for the 58-day course of the experiment, and the number of glands available for examination was too small to permit definite conclusion. The 6-methyl-5-iodo-thiouracil had no effect on either the weight or iodine content of the thyroid.

Some of the changes in thyroid histology produced by the various treatments are shown in the accompanying figures. For the same compounds the changes are quite similar to those shown in the preceding paper. The thyroids of animals treated with thiouracil showed pronounced histological changes after 19 days, although no change was found in the weight of the glands. With regard to the iodinated 6-methyl derivatives of thiouracil it is interesting to note that the thyroid histology indicates some kind of activity, despite the largely negative results obtained by other criteria. Further studies of this compound may be warranted.

DISCUSSION

Since the only appreciable effect of 5-iodothiouracil on the thyroid is a partial depletion of its iodine content, it was significant to find that the antimetabolic potency of this compound in the normal rat is practically equal to that of thiouracil. Williams *et al.* (1949) have reported that the iodo derivative in the hyperthyroid human lowers the BMR more rapidly than does thiouracil; this action resembles somewhat an iodine effect. These workers also reported, in agreement with our findings in the rat, that the 6-methyl analogue was without effect. While 5-iodothiouracil may indeed possess some type of iodide effect which would explain its antithyroid behavior, it is difficult to see why the 6-methyl analogue does not

possess the same effect since their chemical stabilities are quite similar. In view also of further results with 5-iodothiouracil, namely, its "after-effect" in maintaining a depressed metabolism and its dissimilarity from an equivalent mixture of thiouracil and potassium iodide, it seems that the unique antithyroid action of this compound should be ascribed to the entire molecule rather than to any particular moiety. This conclusion would also apply to the other 5-halogeno derivatives.

Although the few metabolic studies with inorganic iodides reported in the literature have not given uniform results, these substances are assumed by a number of investigators to exert some kind of suppressive action on thyroid functioning (Loeser and Thompson, 1934; Salter, 1940; Crepax, 1948; Wolff and Chaikoff, 1948 a, b; Rawson, 1949). The small but consistent minus values in the OC curve of the animals treated with potassium iodide for over a month may represent such a suppression, but this requires repetition. When given with an equimolar amount of thiouracil, any effect of the iodide on OC was completely obscured.

Results so far obtained with the iodothiouracil indicate that it possesses a unique antithyroid action. In equivalent dosages it is much less active than thiouracil in depleting thyroid iodine in the rat but is fully as active as thiouracil in depressing the metabolism. In the hyperthyroid human it is even more effective than thiouracil in lowering the BMR. (Williams *et al.* 1949). However, despite the antimetabolic effect, 5-iodothiouracil does not cause the thyroid hypertrophy with accompanying hyperemia and friability which is produced either by thiouracil or by a thiouracil-potassium iodide mixture. It is considered that these results support the original assumption that an iodinated thiouracil might possess valuable clinical properties.

SUMMARY

Oral administration of equimolar quantities of thiouracil, thiouracil plus potassium iodide, and the 5-halogeno derivatives of thiouracil produced about the same lowering of oxygen consumption in the young Sprague Dawley rat. Potassium iodide alone and 6-methyl-5-iodothiouracil were much less effective. The relative antithyroid potencies of thiouracil and of 6-methylthiouracil were reversed by iodine substitution into the ring. None of these compounds, in quantities up to 78 umols per day per 100 gm. body weight, was as effective as thyroidectomy in lowering oxygen consumption. After cessation of treatment, a prolonged metabolic depression followed use of the 5-iodothiouracil; this effect was not duplicated by an equivalent mixture of thiouracil and potassium iodide. Animals receiving the bromo derivative exhibited toxicity after a month of treatment, but none of the other drugs produced untoward effects. Potassium iodide alone

and also the iodinated derivatives appeared to have some beneficial effect on the growth of the animals under the conditions of this experiment. The 5-iodothiouracil was found to have a unique antithyroid effect in that it depressed the metabolism to the same extent as did thiouracil, partially depleted the thyroid iodine, but produced no measurable thyroid hyperplasia. It is suggested that the unique effect of the iodo derivative is due to the entire molecule rather than to any particular moiety.

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THE INFLUENCE OF SEX AND OF TESTOSTERONE UPON FATTY LIVER DUE TO ETHIONINE[1]

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ETHIONINE (α-amino-γ-ethylmercapto-butyric acid), a presumed antagonist of methionine, had previously been shown to induce a fatty infiltration of the liver in fasted female rats (Farber, Simpson and Tarver, 1950). Methionine prevented the rise in liver lipids and protected the animals against death while other amino acids, as well as choline, inositol and dimethylthetin were ineffective. Glucose, in large doses, also prevented and cured the fatty change, but failed to prevent the death of the rats. A specific ethionine-methionine antagonism was indicated.

During these studies it was observed that, while fasted females readily developed the fatty liver, fasted male rats were refractory. The results of a more extensive investigation of this sex difference as well as of the effects of administration of testosterone propionate to both females and castrated males are here reported. In addition, a similar sex difference in mortality of ethionine-treated rats is presented.

EXPERIMENT

Albino rats, all of the same strain, were used. For the investigations on liver lipids the virgin females weighed from 170 to 250 gm., while the males ranged from 190 to 300 gm. For the mortality experiments, the weights of the virgin females and males were as indicated in Table 4. Prior to the experiment, the animals were fed a commercial diet.[4] The animals were fasted for a period of 12 to 16 hours before the first administration of ethionine, and thereafter until sacrificed. Water was available at all times.

The DL-Ethionine was administered intraperitoneally in aqueous solution (25 mg. per cc.) after neutralization with $NaHCO_3$. In the experiments on liver fat, experimental animals received 1 gm. of ethionine per gm. initial body weight in three divided doses (every $2\frac{1}{2}$ hours) and were sacrificed 24 hours after the first dose, with the excep-

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tion of one group reported in Table 1, which was killed after 48 hours. The control groups received an equal volume of distilled water. Testosterone propionate in sesame oil was injected subcutaneously in doses of 1 mg., 2 mg., and 5 mg. once daily for three days prior to and twice on the day of administration of ethionine. In some experiments the hormone was also given on alternate days for periods of 7 to 9 days. For the studies on mortality rate, the dosage of ethionine was as indicated in Table 4.

For liver fat analysis, the animals were killed under ether anesthesia after withdrawal of blood from the abdominal aorta. Aliquots of the liver tissue were dried by grinding with anhydrous sodium sulfate and extracted with chloroform for 24 hours, as described earlier (Farber, Simpson and Tarver, 1950). The residue from this extract was dissolved in petroleum ether and the total lipids were determined gravimetrically, following the evaporation of the solvent. Because of the marked increase in liver weights in the experimental group, the total liver lipids were expressed in both per cent wet weight of liver and mg. per 100 gm. initial body weight. Bromsulphalein retention was determined by analysis of the plasma dye concentration 30 minutes after the intraperitoneal injection of 5 mg. of dye per 100 gm. body weight (Casals and Olitsky, 1946). Ovariectomy was performed at an age of 2 to 3 months (140 to 160 gm.) and orchidectomy at the same age (150 to 170 gm.). Pieces of liver were fixed in 10 per cent neutral buffered formalin. Paraffin sections were stained with hematoxylin eosin and frozen sections were stained with Sudan IV.

RESULTS

The total liver lipids following the administration of ethionine were considerably higher in female than in male rats (Table 1). The value for

TABLE 1. TOTAL LIVER LIPIDS AND BROMSULPHALEIN RETENTION IN MALE AND FEMALE RATS WITH OR WITHOUT ETHIONINE ADMINISTRATION

No. of rats	Sex	Group	Duration of experiment	Total liver lipids		Bromsulphalein
			hours	mg./100 gm. wet liver	mg./100 gm. initial body weight	mg./100 cc plasma
17	f	Control	24	5.5 ±0.2*	139 ± 7*	0.29 ±0.10*
26	f	Ethionine	24	14.8 ±0.6	475 ±20	2.74 ±0.13
15	m	Control	24	5.2 ±0.2	139 ± 8	0.24 ±0.07
45	m	Ethionine	24	6.2 ±0.2	190 ± 6	0.50 ±0.06
7	f & m	Control	48	5.3 ±0.2	128 ± 7	—
12	f	Ethionine	48	19.4 ±1.7	682 ±23	—
6	m	Ethionine	48	6.2 ±0.2	149 ± 8	—

* Standard error of the mean.

the males was slightly higher than for the controls. The sex difference persisted for at least 48 hours. These differences in total liver lipids at both time intervals are statistically significant according to the standard error of the mean. Overlap was exceptional. Only 2 males had total liver lipids of over 9 gm. per 100 gm. liver (9.7) while the lowest for the females were 8.2 gm. in one and 10.7 gm. in another; all other females showed values above 12 gm. The bromsulphalein retention mirrored the increase in liver

fat, as has been recently reported for fatty livers with and without necrosis (Koch-Weser *et al.*, 1950). The male and female control animals had almost identical values for liver fat.

Ovariectomy did not protect the female rats against fatty liver and bromsulphalein retention due to ethionine (Table 2) while orchidec-

TABLE 2. TOTAL LIVER LIPIDS AND BROMSULPHALEIN RETENTION IN NORMAL AND OVARIECTOMIZED FEMALE RATS WITH OR WITHOUT PRECEDING ETHIONINE AND TESTOSTERONE PROPIONATE ADMINISTRATION

No. of rats	Group	Ovariectomy	Postoperative period	Testosterone propionate		Total liver lipids		Bromsulphalein
			days	mg.	days	in mg./100 gm. wet liver	in mg./100 gm. initial body weight	in mg./100 cc. plasma
6	Control	+	40	—	—	5.6±0.3	150± 9	0.08±0.02
7	Ethionine	+	40	—	—	15.5±1.4	491±42	1.82±0.24
7	Ethionine	—	—	20*	4	16.3±1.2	553±48	2.11±0.28
19	Ethionine	—	—	16†	11	5.8±0.3	173± 2	0.61±0.15
20	Ethionine	+	40	16†	11	6.1±1.0	169±10	0.63±0.12

* 5 mg. once daily
† 2 mg. on alternate days for 8 days, followed by 2 mg. once daily for 2 days and 2 mg. twice on the day of ethionine administration.

tomized males became susceptible (Table 3). This was clearly apparent in animals which had been castrated more than a month before the experiments. In those castrated only two weeks prior to the experimental

TABLE 3. TOTAL LIVER LIPIDS AND BROMSULPHALEIN RETENTION IN ORCHIDECTOMIZED MALES WITH AND WITHOUT ETHIONINE AND TESTOSTERONE PROPIONATE ADMINISTRATION

No. of rats	Group	Postoperative period	Testosterone propionate		Total liver lipids		Bromsulphalein
		days	mg.	days	in mg./100 gm. wet liver	in mg./100 gm. initial body weight	in mg./100 cc. plasma
10	Control	14	—	—	3.8±0.3	90±10	0.05±0.01
15	Ethionine	14	—	—	7.8±0.4	228±12	2.14±0.30
13	Control	38	—	—	5.3±0.2	120± 5	0.13±0.02
9	Ethionine	38	—	—	12.8±0.9	353±31	2.09±0.30
13	Ethionine	14	5	4	4.5±0.2	119± 6	0.32±0.05
10	Ethionine	38	5	4	10.8±0.7	284±19	2.24±0.33
10	Ethionine	38	9*	12	5.2±0.2	139± 7	—

* 1 mg. on alternate days for 8 days, followed by 1 mg. once daily for 4 days

period the total lipids were lower in both controls and ethionine treated animals, the difference between the two groups being similar to that between other groups. In general, the males showed a greater variability in the liver lipids than the females.

The administration of testosterone propionate to intact females prevented the rise in liver lipids, but only when injected for a period of 11 days (Table 2). Despite large amounts of administered hormone, no protection was observed after a 4-day treatment. Administration for longer periods also protected ovariectomized females.

Castrated males reacted like females (Table 3). After a postoperative

period of two weeks, testosterone propionate was effective when administered for only 4 days. However, in males orchidectomized 38 days previously, a 4 day period of treatment was insufficient for complete protection. The longer period of hormone administration effected a complete reversal as in females.

In the operated males, the response of the liver lipids and seminal vesicles and prostate were frequently not quantitatively parallel. For example, in the animals castrated for the longer interval, a 4 day treatment with testosterone propionate resulted in a marked enlargement of the seminal vesicles and prostate while only a very incomplete effect was observed on the fatty changes of the liver.

The relation between the concentration of lipids and their total amount, although in essential agreement in most cases, did show noticeable dis-

TABLE 4. MORTALITY OF MALE AND FEMALE RATS 72 HOURS AFTER THE INTRAPERITONEAL ADMINISTRATION OF 1.5 MG. OF ETHIONINE PER 100 GM. INITIAL BODY WEIGHT

No. of rats	Sex	Weight range in gm.	Number dead
14	f	162–333	14
21	m	210–366	1

crepancies. For example, in one intact female the liver had a concentration of 8.2 gm. fat per 100 gm. wet tissue (a value intermediate between controls and experimental) while it contained 400 mg. per 100 gm. initial body weight; the latter value is within the experimental range for this group.

The females showed, following ethionine administration, a mortality rate of 100 per cent in 72 hours, while of the 21 males, only one succumbed (Table 4). The average weight of the males was somewhat higher than that of the females; their ages, however, were comparable and females of all weights showed the same mortality. The males survived even after 144 hours. In a number of unpublished experiments 50 fasted control male and female rats, injected with distilled water alone, showed 100 per cent survival rates in both sexes at the above time intervals.

Histological examination of the livers confirmed the chemical findings. In the females and untreated castrated males, large amounts of sudanophilic material were seen, especially in the peripheral parts of the liver lobule. The fat decreased in amount from the periportal to the central areas. No large areas of necrosis were present in any of the sections. Some sections did show very small (usually 1 to 3 cells) scattered areas of focal necrosis, with no uniform distribution. These focal areas of eosinophilic necrosis were infiltrated with a few histiocytes and polymorphonuclear leucocytes.

DISCUSSION

A clear cut sex difference in the response of the liver to the administration of ethionine in rats has been demonstrated by the rise in the total liver lipids and the parallel bromsulphalein retention, the latter apparently caused by the fat accumulation (Koch-Weser *et al.* 1950).

The lack of response of the males seems to be mediated by an androgen. Testosterone propionate protects castrated males and intact and spayed female rats against fatty metamorphosis of the liver produced by ethionine. However, a time factor is involved since the administration of the androgen produced, after short term treatment, enlargement of prostate and seminal vesicles, but afforded little protection of the liver against the ethionine effect.

A sex difference in fat metabolism has been known for some years. Deuel and Gulick (1932) found that fasted female rats exhibited a greater degree of ketosis than did males. Estrogens produce a rapid rise in blood and liver lipids in fowl (Lorenz, Chaikoff and Entenman, 1938; Lorenz, Entenman and Chaikoff, 1939) and increase the serum lipid concentration and fat storage in rats under special dietary conditions (Loeb, 1942 a, 1942 b). More fat accumulates in the liver of female rats during the first 24 hours after partial hepatectomy (MacKay and Carne, 1938); ovariectomy decreases this fatty infiltration (Szego and Roberts, 1949). In addition, Gyorgy, Rose and Shipley (1947, 1949) have assigned a lipotropic role to estrone and other estrogens; ovariectomy favors the development of fatty livers in rats on a low protein high fat diet while castration in the male is without effect (Shipley, Chudzik and Gyorgy, 1948).

The results of the present investigation tend to implicate the androgens also in fat metabolism. The mechanism of the protective effect of testosterone propionate in the ethionine fatty liver requires explanation.

The possibility of a more rapid renal excretion of ethionine due to the known structural sex differences of the kidney (Selye, 1939), is refuted by studies on the influence of ethionine on the transfer of the methyl group of methionine to the choline containing phospholipids in the liver and on the conversion of the methyl group to formate. Ethionine inhibits transmethylation from methionine (labeled with C^{14} in the methyl group) in intact male rats, castrated males, and females *in vivo* to an equal degree (Farber, 1949). Also, the conversion of the methyl group of methionine to formate is equally depressed in the liver of intact males and females (Siekevitz, 1949). These findings indicate that ethionine is entering the liver cell in sufficient concentration to inhibit at least these reactions.

Against the possibility that testosterone propionate influences the removal of fat from rather than its infiltration into the liver speaks the ob-

servation that the administration of large doses of the hormone for 4 days had no influence upon the total liver lipids in ethionine-treated females.

It appears more attractive to assume an effect of the androgen not directly upon lipid but rather on protein metabolism. Ethionine depresses the rate of incorporation of radioactive methionine (S^{35}) and glycine (C^{14}) into the liver proteins in intact female rats, a process identified with protein synthesis (Simpson, Farber and Tarver, 1950). Male rats showed no such inhibition (Farber, 1949, Tarver, personal communication). While the influence of testosterone in females upon this incorporation has not yet been investigated, it is possible that this sex difference may also be mediated through androgens.

The coincidence of fatty liver and inhibition of protein synthesis suggests an etiological link. Since the protein changes appear before the increase in liver lipids (Farber, Simpson and Tarver, 1950), the former seems to be primary. A similar relationship between disturbances in protein metabolism and fatty livers in rats maintained on a low protein diet has been suggested (Jaffe *et al.*, 1949). Further support for this contention is offered by the work of Adamstone and Spector (1950) who demonstrated fatty changes in the livers of rats forced-fed a tryptophane-deficient diet. Rats maintained on a similar diet incorporate radioactive tryptophane (C^{14}) and glycine (C^{14}) into the liver proteins *in vivo* at a diminished rate (Sanadi and Greenberg, 1948). This suspected relation between the disturbance in protein metabolism and the infiltration of fat in the liver is under study. Both methionine (Miller, 1944, Brush, Willman and Swanson, 1947) and testosterone propionate (Kochakian, 1944) possess a nitrogen-sparing action in rats. The effect of this hormone in opposing an antagonist of methionine suggests a possible link between the androgen and the amino acid.

If the fatty liver due to ethionine is related to disturbed protein metabolism, the effect of sex and testosterone is possibly due to the established nitrogen-sparing effect of testosterone. The latter may reduce the protein requirements of the liver. The observation that the pancreatic lesions produced by ethionine reveals no sex difference (Farber and Popper, 1950) is possibly explained by the greater protein formation of the pancreas in comparison to the liver (per unit weight) which makes the former organ vulnerable despite the protein sparing effect of testosterone.

The reason for the death of ethionine-treated animals is still uncertain. The fatty liver *per se* is not responsible for the death. The administration of large doses of glucose either prevents the fatty infiltration of the liver due to ethionine or removes the fat after its accumulation (Farber, Simpson and Tarver, 1950), this despite the fact that this carbohydrate had no influence upon the inhibition of protein synthesis (Simpson *et al.*, 1950).

The lethal dose of ethionine is larger than the one required to produce a fatty liver (Koch-Weser *et al.*, 1950). The latter may disappear following sublethal doses. This indicates quantitative differences in the ethionine effect and is also in keeping with the possibility that the lethal effect of ethionine is mediated through an interference with protein synthesis in the animal organism in general. The previously suggested protein-sparing effect of testosterone may also apply here.

SUMMARY

A sex difference in the occurrence of fatty liver and bromsulphalein retention produced by ethionine (a metabolite antagonist of methionine) in rats has been demonstrated, the males being refractory. Orchidectomized males lost their resistance to ethionine, especially more than 14 days after castration. Ovariectomy does not influence the liver changes. Testosterone propionate, when administered for a period of 11 days, protected both females and castrated males against the fatty infiltration. The androgens, therefore, appear to be responsible for the sex difference. A similar sex variation has also been demonstrated in the lethal effect of ethionine. The sex differences have been associated with the protein-sparing effect of testosterone and a possible relation between methionine and testosterone has been suggested.

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THE EFFECT OF CORTISONE AND THE ADRENOCORTICOTROPHIC HORMONE OF THE PITUITARY ON THE BLOOD PRESSURE IN EXPERIMENTAL RENAL HYPERTENSION[1]

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THE existence of a relationship between the adrenal cortex and the hypertensive state has long been conjectured (Grollman, 1936; Grollman, Harrison and Williams, 1940; Perera, 1950). Despite the many observations suggestive of the existence of such a relationship, the available data fail to support such a view. The recent interest in cortisone and the adrenocorticotrophic hormone of the pituitary (ACTH) has again aroused interest in this problem and led to the trial of these drugs in clinical hypertension. Because of the difficulties and uncertainties involved in evaluating changes observed in the blood pressure of the human subject, the effects of the above-mentioned drugs on the blood pressure in experimental renal hypertension was determined.

MATERIAL AND METHODS

Rats and dogs rendered hypertensive by application of a figure-of-eight ligature to one kidney and removal of the contralateral one (Grollman, 1944) were injected intramuscularly with cortisone and ACTH. Cortisone (Merck) was given in doses of 1 and 2 mgms. daily to the rats and in doses of 50 mgms. daily to the dogs. ACTH was administered in doses of 1 mgm. daily to the rats and in doses of 2 mgms. per kilo of body weight daily to the dogs. The preparation used was corticotropin (Wilson), which, according to the manufacturer's assay, had an activity per mgm. equivalent to 2.5 times the La-1-A standard.

The blood pressures in the rats were determined once daily, before, during and following the period of the injections, by the plethysmographic method of Williams, Harrison and Grollman (1939). The mean blood pressures of the dogs were determined by direct puncture of the femoral artery as previously described (Grollman, 1946).

RESULTS

The results of the present study as obtained on hypertensive rats is shown in Fig. 1. As noted from this figure cortisone (upper curve) and ACTH (middle curve) induce only slight depressions in blood pressure

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when used in doses comparable to those ordinarily used in the human. The results reproduced in Fig. 1 were obtained on a dosage level of 2 mgms.; on a dosage of only 1 mgm. daily, the resulting changes in blood pressure were within the limits of the normal variations in daily blood pressure readings.

The results on the hypertensive dogs are shown in Table 1 and are entirely comparable to those obtained in the rat with a maximum average lowering of the blood pressure of only 10 mms. Hg. Control studies on normotensive rats and dogs using the same dosages of cortisone and ACTH as

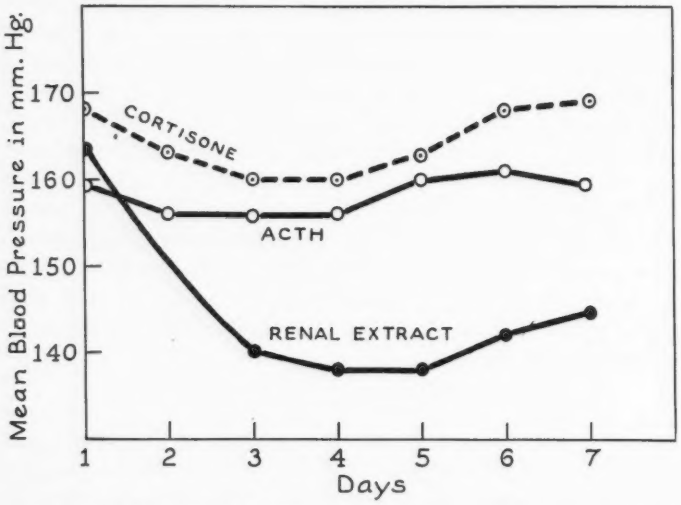

FIG. 1. The effects of cortisone, ACTH and renal extract on the blood pressure of series of 12 hypertensive rats. The rats treated with cortisone (uppermost curve) received 2 mgms. daily intramuscularly for the first 3 days of the experimental period. Those receiving ACTH (middle curve) received 2 mgms. daily for a similar period. The lower curve is based on an experiment in which a purified renal extract was administered orally mixed with the animal's regular food during the first day of the experimental period.

in the experimental groups induced no changes greater than the normal daily variations.

For the purposes of comparison the lower curve of Fig. 1 is included to show the response of a group of 12 rats to the oral administration of a purified preparation of renal extract. This was administered mixed in the animals' regular food for the first day of the experiment. The curve shows the striking and sustained reduction in blood pressure induced by renal extract in comparison with the feeble effects of cortisone and ACTH. The extract used was a highly purified one with a total solid content of less than 100 mgms. per cc. which was the dose administered per rat.

DISCUSSION

The results of the present study demonstrate the ineffectiveness of cortisone or ACTH in the therapy of experimental hypertension which is in confirmation of the preliminary results thus far obtained in the human. The absence of response is in accord with other data indicating that hypertension in the human as well as in the experimental animal is a result of renal dysfunction rather than a response to stress or neurogenic disturbances acting through the adrenal or pituitary.

In the enthusiasm accompanying the recent demonstration of the clinical use of cortisone and ACTH, it has been overlooked that the positive therapeutic effects in disease induced by unphysiological doses need not necessarily imply that these conditions are induced by hormonal imbalances. With further experience in the many disease entities which have been treated by these drugs it has become apparent that no simple rela-

TABLE 1. THE EFFECT OF CORTISONE AND ACTH ON THE BLOOD PRESSURE OF THE HYPERTENSIVE DOG. EACH VALUE GIVEN IS THE AVERAGE OF THE DAILY DETERMINATION ON GROUPS OF 3 ANIMALS USED IN EACH EXPERIMENT. INJECTIONS WERE MADE ONCE DAILY FOR THE FIRST 3 DAYS OF THE EXPERIMENTAL PERIOD

Day	1	2	3	4	5	6	7
	Mean blood pressure in mm. Hg.						
Cortisone	170	170	160	160	155	160	170
ACTH	165	170	160	160	165	165	170

tionship exists between adreno-cortical and pituitary function and the disease in question. This is illustrated, for example, in the recent study of Gutman and Yü (1950) who, contrary to the earlier report that ACTH was specific for the treatment of gout, conclude that "the available evidence does not appear to justify the view that the pituitary and/or adrenal glands plays a central role in the pathogenesis of gout." Likewise, in the case of acute rheumatic fever, there is no evidence (Hain and Wilson, 1949) of a deficiency in the adrenocortical reserves despite the apparent beneficent effects of cortisone and ACTH in this disorder.

The results obtained in the present study are in variance with those of Perera (1950) who noted an increase in blood pressure in most of his hypertensive patients receiving ACTH but a decline in those receiving cortisone. However, in view of the frequency with which sodium retention and other effects are induced by ACTH and cortisone, it is probable that the observed rises, as well as the falls, are secondary effects induced by these substances (Sprague, *et al.*, 1950). The psychic reactions to ACTH and cortisone, for example, might induce a rise in blood pressure in the human, not elicited in the animal.

In view of the negligible effects of ACTH and cortisone on the blood pressure, it is improbable that the effect of a low-protein diet in reducing the blood pressure in experimental nephritis is a result of a decreased ACTH output (Handler and Bernheim, 1950).

The slight effects of the two drugs on the blood pressure in the normotensive as well as in the hypertensive as compared to the striking effects of salt depletion in the latter also make it improbable that the effects of sodium depletion are mediated through the pituitary-adrenal axis as suggested by Selye (1948).

SUMMARY

The effects of cortisone and ACTH on the blood pressure of hypertensive rats and dogs is so slight as to be attributable to the pharmacodynamic effects induced by these drugs rather than to any specific effect on the disorder. The results obtained in this study as well as the other available data fail to support the view that hypertension is a result of adrenal-cortical or pituitary dysfunction induced by stress or nervous influences.

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THE EFFECTS OF CONTINUOUS LIGHT OR DARKNESS ON THYROID FUNCTION IN MICE[1,2]

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SEASONAL fluctuations in thyroid function have usually been attributed to temperature changes because of the demonstrated action of heat and cold on thyroid activity. Although the effects of seasonal changes in light-increment on thyroid secretion have not yet been investigated, it seems possible that this too may be a factor. It is also possible that some of the reported effects of light on gonadal activity may have been due, in part at least, to light-induced changes in thyroid secretion. That alterations in thyroid function can induce profound changes in gonadal activity has been emphasized in recent reports by Meites *et al.* (1949) and Maqsood *et al.* (1950).

The available data on the effects of light on thyroid function are not in agreement. Bergfeld (1930) stated that absence of ultraviolet light caused thyroid hypertrophy in rats irrespective of environmental temperature. In twelve female rats which were kept in continuous light for one month, Dempsey (1943) noted a reduction in thyroid cell height. However, Kenyon (1935) and Mayerson (1935) reported that in their experiments neither darkness nor light influenced thyroid activity in rats. In chicks, Turner and Benedict (1932) claimed that absence of ultraviolet light resulted in marked thyroid hypertrophy and colloid loss, while Kleinpeter and Mixner (1947) reported that increased quantities of light slightly increased thyroid activity when compared to controls kept under normal light conditions.

The objective of the research reported here was to determine whether or not any conclusive relationship could be demonstrated between the extremes of continuous presence of light or complete absence of light and thyroid secretion in mice. This was done by placing mice under continuous light or darkness for twenty-eight days, and then measuring thyroid

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activity by determining (1) the reaction of the thyroids to thiouracil injections (2) the normal changes in thyroid weight and (3) the uptake by the thyroids of a tracer dose of radioactive iodine (I^{131}).

METHODS

Rockland albino mice of both sexes were divided into uniform groups (by weight and sex) of ten each, and were placed in ample-sized screen cages for twenty-eight days, at the end of which period they were sacrificed. A fifteen watt light bulb was placed about four inches in front of each cage which was to be maintained under continuous light. The cages of the animals kept in continuous darkness were completely covered, except that the covers were not tight enough to exclude air circulation. Control groups of animals were kept under the day-night lighting conditions prevailing in the animal room, or on about nine hours of light and fifteen hours of darkness daily. Food and water were available at all times.

The experiments were conducted in an air conditioned animal room at a mean temperature of 75° F. Numerous checks with a thermometer were made during the course of the experiments and not more than one-half degree variation in temperature was found among the different cages. It is believed, therefore, that temperature can be excluded as a factor in these experiments.

The thiouracil-treated mice were each injected subcutaneously with ten mg. of the drug daily during the last ten days of the twenty-eight day experimental period. The drug was injected rather than fed in order to insure that each animal received the same quantity of the material. On the day of sacrifice the thyroids were carefully removed and weighed on a Roller-Smith balance.

In the experiments in which radioactive iodine was used as an indicator of thyroid activity, each animal was injected intraperitoneally, sixteen hours prior to sacrifice, with a tracer dose of I^{131} containing approximately 0.1 microcurie of activity. After the animals were killed, the thyroids were removed, weighed, permitted to dry overnight and then placed in small copper discs for counting. Each thyroid was counted separately under a thin mica end window counter.

The experimental data were treated statistically. Standard errors of the means and significant differences between means were determined by the usual formulas.

RESULTS

Female Mice—Thiouracil Action. The results of two preliminary experiments are given in Table 1. It can be seen that the thyroids of the mice which were under continuous light for twenty-eight days (groups 2) showed a reduced response to thiouracil, and hence weighed significantly less than the controls which were exposed to the customary day-night lighting in the animal room. In the next experiment (Table 2) thiouracil given to mice under control conditions (group 2) increased the average thyroid weight to 24.49 ± 2.64 mg. as compared to 10.09 ± 0.60 mg. in the non-thiouracil treated mice (group 1). Continuous darkness significantly increased the thyroid response to thiouracil (group 3) while continuous light reduced the response to thiouracil (group 4).

TABLE 1. EFFECT OF CONTINUOUS LIGHT ON THIOURACIL ACTION IN FEMALE MICE

Group	No. per group	Treatment	Ave. original body wt. gm.	Av. final body wt. gm.	Ave. thyroid wt. mg.	Ave. *thyroid wt* 100 gm. body wt. mg.
1	9	Controls plus thiouracil	28.00	31.33	6.99 ± 0.52*	22.31 ± 1.27*
2	10	Continuous light plus thiouracil	28.70	32.75	4.94 ± 0.33*	15.10 ± 1.00* Significant difference = 4.45
1	10	Controls plus thiouracil	25.00	26.00	6.20 ± 0.60*	24.06 ± 1.82*
2	9	Continuous light plus thiouracil	23.80	23.38	3.79 ± 0.69*	16.10 ± 2.27* Significant difference = 2.74

* Standard error of mean.

Female Mice—Thyroid Uptake of Radioactive Iodine. The results of this experiment (Table 3) confirm the data obtained in the thiouracil-treated mice. In the upper half of the table it can be seen that the thyroids of the animals under continuous darkness (group 2) weighed more, while under continuous light (group 3) they weighed less than in the controls (group 1). It is not believed that the single injection of I^{131} affected the weights of these thyroids. The uptake of radioactive iodine by the thyroids of the mice under continuous darkness (group 2) was significantly greater than by the thyroids of the controls (group 1), whether calculated on a total thyroid, per mg. of thyroid or on a body weight basis. On the other hand, the thyroids of the mice which had been under continuous light (group 3) took up significantly less I^{131} than the controls (group 1).

TABLE 2. EFFECTS OF CONTINUOUS LIGHT OR DARKNESS ON THIOURACIL ACTION IN FEMALE MICE

Group	No. per group	Treatment	Av. original body wt. gm.	Av. final body wt. gm.	Av. thyroid wt. mg.	Av. *thyroid wt.* 100 gm. body wt. mg.
1	9	Controls	22.70	27.12	2.70 ± 0.11*	10.09 ± 0.60*
2	10	Controls plus thiouracil	22.70	26.31	6.35 ± 0.59*	24.49 ± 2.64*
3	9	Continuous darkness plus thiouracil	22.90	25.46	8.01 ± 0.69*	31.18 ± 2.01*
4	9	Continuous light plus thiouracil	22.90	25.56	3.87 ± 0.58*	15.11 ± 2.37* Significant differences: Groups 2 and 3 = 2.02 Groups 2 and 4 = 2.64

* Standard error of mean.

TABLE 3. EFFECTS OF CONTINUOUS LIGHT OR DARKNESS ON THE UPTAKE OF RADIOACTIVE IODINE IN FEMALE MICE

Group	No. per group	Treatment	Av. orig. body wt. gm.	Av. final body wt. gm.	Ave. thyroid wt. mg.	Av. *thyroid wt.* 100 gm. body wt. mg.
1	10	Controls	23.70	28.88	2.53 ± 0.11*	8.76 ± 0.36*
2	10	Continuous darkness	24.00	27.94	2.87 ± 0.11*	10.27 ± 0.69*
3	10	Continuous light	24.00	27.53	2.14 ± 0.14*	7.77 ± 0.25*

Group	Radioactivity in counts per second		
	Ave. no. counts per thyroid	Ave. no. counts per mg. thyroid	Av. no. counts per thyroid 100 gm. body wt.
1	21.57 ± 1.72*	8.74 ± 0.38*	75.44 ± 6.59*
2	28.23 ± 2.52*	9.89 ± 0.42*	99.76 ± 6.95*
3	12.39 ± 1.45*	6.01 ± 0.30*	44.21 ± 4.15*
	Significant differences: Groups 1 and 2 = 2.18 1 and 3 = 4.08	Significant differences: Groups 1 and 2 = 2.04 1 and 3 = 5.65	Significant differences: Groups 1 and 2 = 2.54 1 and 3 = 4.01

* Standard error of mean.

Male Mice—Thiouracil Action. The results of these two experiments (Tables 4 and 5) are essentially similar to those obtained in the female mice. In both experiments, continuous darkness (group 3) significantly augmented the action of thiouracil on the thyroid, while continuous light (group 4) reduced the effect of thiouracil when compared to the controls (group 2).

TABLE 4. EFFECTS OF CONTINUOUS LIGHT OR DARKNESS ON THIOURACIL ACTION IN MALE MICE

Group	No. per group	Treatment	Av. original body wt. gm.	Av. final body wt. gm.	Av. thyroid wt. mg.	Ave. thyroid wt. 100 gm. body wt. mg.
1	10	Controls	32.00	35.10	2.55 ± 0.15*	7.23 ± 0.29*
2	8	Controls plus thiouracil	33.90	38.69	8.36 ± 0.59*	21.71 ± 1.04*
3	6	Continuous darkness plus thiouracil	33.60	37.17	10.33 ± 0.99*	28.10 ± 2.36*
4	10	Continuous light plus thiouracil	32.00	33.01	4.60 ± 0.50*	14.64 ± 1.17*
						Significant differences: Groups 2 and 3 = 2.29 Groups 2 and 4 = 3.74

* Standard error of mean.

TABLE 5. EFFECTS OF CONTINUOUS LIGHT OR DARKNESS ON THIOURACIL ACTION IN MALE MICE

Group	No. per group	Treatment	Av. original body wt. gm.	Av. final body wt. gm.	Av. thyroid wt. mg.	Ave. thyroid wt. 100 gm. body wt. mg.
1	10	Controls	21.40	32.06	2.91 ±0.34*	8.92 ±0.81*
2	8	Controls plus thiouracil	21.50	31.06	6.29 ±0.61*	20.74 ±2.35*
3	9	Continuous darkness plus thiouracil	21.40	29.27	7.91 ±0.47*	27.28 ±1.30*
4	10	Continuous light plus thiouracil	21.50	31.01	4.61 ±0.49*	14.65 ±1.18*
						Significant differences: Groups 2 and 3 = 2.43 Groups 2 and 4 = 2.32

* Standard error of mean.

Male Mice—Thyroid Uptake of Radioactive Iodine. The data obtained in this experiment (Table 6) corroborate the results obtained with thiouracil. Continuous darkness (group 2 in upper half of table) produced an increase and continuous light (group 3) a decrease in thyroid weight when compared to the controls (group 1). The uptake of I^{131} by the thyroids of the

TABLE 6. EFFECTS OF CONTINUOUS LIGHT OR DARKNESS ON THE UPTAKE OF RADIOACTIVE IODINE IN MALE MICE

Group	No. per group	treatment	Av. original body wt. gm.	Av. final body wt. gm.	Ave. thyroid wt. mg.	Ave. thyroid wt. 100 gm. body wt. mg.
1	9	Controls	21.80	31.49	2.19 ±0.05*	6.95 ±0.43*
2	10	Continuous darkness	21.40	31.76	2.62 ±0.21*	8.25 ±0.61*
3	9	Continuous light	21.80	31.67	1.93 ±0.12*	6.09 ±0.36*

Group	Radioactivity in counts per second: Av. no. counts per thyroid	Ave. no. counts per mg. thyroid	Ave. no. counts per thyroid 100 gm. body wt.
1	22.60 ±2.97*	10.19 ±0.46*	74.59 ±10.48*
2	31.03 ±3.00*	11.51 ±0.47*	117.43 ±12.35*
3	13.70 ±2.26*	6.90 ±0.78*	41.77 ±6.06*
	Significant differences: Groups 1 and 2 = 2.00 1 and 3 = 2.30	Significant differences: Groups 1 and 2 = 2.00 1 and 3 = 3.64	Significant differences: Groups 1 and 2 = 2.65 1 and 3 = 2.71

* Standard error of mean.

mice under continuous darkness (group 2 in lower half of table) was significantly more and under continuous light (group 3) was significantly less than in the controls (group 1).

DISCUSSION

Three kinds of evidence substantiate the conclusion that continuous light reduces thyroid function and that continuous darkness increases thyroid function in Rockland mice of both sexes.

Thiouracil was used in most of the measurements of thyroid function in these experiments. It was assumed at the outset that if each animal were injected with the same dose of thiouracil, there should have been the same average increase in thyroid weight of each animal. Thyrotropic activity by the anterior pituitary would presumably be stimulated to the same degree in all animals. However, if continuous light induced an initially lower level of thyrotropic and thyroid function, then the administration of thiouracil might be expected to produce a smaller increase in thyroid weight. Under continuous darkness, an initially higher level of thyrotropic and thyroid activity would be produced, with a subsequently greater increase in thyroid weight through thiouracil action.

In the male and female mice which were to be injected with a tracer dose of radioactive I^{131}, continuous light resulted in a decrease and continuous darkness in an increase in thyroid weight. It is not believed that the single injection of I^{131} influenced thyroid weight in these animals. Although changes in thyroid weight do not always represent changes in function, it appears logical to make this conclusion insofar as these experiments are concerned, since they are in agreement with the other measures of thyroid function.

Insofar as the "uptake" of radioactive iodine is concerned, it is believed that a more active thyroid would take up more and a less active thyroid less of the iodine. It is recognized that not all the I^{131} found in the thyroid necessarily represents "uptake" exclusively, since some of the iodine present may only indicate retention and slow excretion from the gland. However, it seems doubtful that the latter would be major factors in the time interval (sixteen hours) which elapsed between the injection of the I^{131} and sacrifice of the mice. Furthermore, these tests of thyroid activity with radioactive iodine are entirely in agreement with the other two measures of thyroid function used in these experiments.

It remains to be explained how light or darkness influenced thyroid activity in the mice. Several interpretations seem possible. Light, through direct stimulation of the eyes, may send nervous impulses to the anterior

pituitary which would induce a decrease in thyrotropic secretion. The absence of light, or darkness, would presumably remove the light-induced inhibition of thyrotropic function.

An alternative hypothesis would give primacy to the effects of light on FSH (follicle stimulating hormone) function by the anterior pituitary. It has been established that FSH and thyrotropic hormone are both elaborated by the same cell type in the anterior pituitary, namely the basophils (Severinghaus, 1939). Thus, if the primary effect of light is to increase FSH production by the anterior pituitary, then the subsequent or secondary effect would be to decrease thyrotropic hormone synthesis. Contrawise, under continuous darkness, FSH synthesis by the basophils would be reduced, thereby permitting more thyrotropic hormone to be secreted.

Another factor which may be important in explaining the results obtained in the mice is the possible influence of continuous light or darkness on general body activity. Mice, like rats, are believed to be more active at night than during the day. It is difficult to determine how much of a factor general body activity may have been in these experiments. If the mice under continuous darkness or light did exercise more and less respectively, there is no indication of this in their final body weights.

The finding that continuous light reduces thyroid activity in mice is in agreement with the report of Dempsey (1943) in female rats. Further work will need to be done to determine whether light and darkness similarly influence thyroid function in other species. It seems reasonable to assume, on the basis of the present work, that seasonal changes in light as well as in temperature probably influence thyroid secretion in mice. Some preliminary work in this laboratory indicates that the changes in thyroid activity induced by light or darkness also modify the gonadal reaction to injections of pregnant mares' serum.

SUMMARY

The effects of continuous light or darkness for twenty-eight days on thyroid activity were studied in 204 albino mice of both sexes. Continuous light induced significant reductions in (a) thyroid weight (b) thyroid reaction to thiouracil and (c) thyroid uptake of radioactive iodine (I^{131}). Continuous darkness produced significant increases in (a) thyroid weight (b) thyroid reaction to thiouracil and (c) thyroid uptake of radioactive iodine. It was concluded, therefore, that continuous light depresses while continuous darkness increases thyroid secretion in mice. Several hypotheses are offered to explain these results.

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A COMPARISON OF URINARY ESTROGENS DETERMINED BIOLOGICALLY AND OF "ESTROIDS" DETERMINED CHEMICALLY*

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PREVIOUS reports from this laboratory (Salter, Humm and Oesterling, 1948; Humm and Salter, 1948) have suggested that the Kober reaction, slightly modified from Venning's procedure (Venning, Evelyn, Harkness and Browne, 1937), could be applied to 6-hour samples of urine in the study of physiological and clinical problems. The question has been raised repeatedly how such chemically determined values correlate with biological assays of urinary estrogens. As emphasized by Engel *et al.* (1950), fluorometric determinations of urinary "estroids" indicate that urine may contain a metabolite which has no estrogenic potency, even though it registers in the fluorometric determination. It is the purpose of the present report to compare the results obtained, colorimetrically and biologically, with the same urine extracts from several distinct groups of individuals. Of special interest is the question whether the low "estroid" excretion in nutritional gynecomastia previously described by Salter, Klatskin and Humm (1947) would be reflected also in a low excretion of estrogens as evaluated biologically. The chief object of this study, however, is to evaluate the "physiological" range of estrogen excretion.

EXPERIMENTAL PLAN

Because colorimetric determinations of "estroids" in pregnancy urine have been feasible for several years (Stevenson and Marrian, 1947; Stimmel, 1946), the studies reported herewith were restricted to lower levels of

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estrogen metabolism. At the upper limit of estrogen excretion a few pregnant women at 3 to 4 months of gestation‡ were included. Most of the individuals, however, were male. Three groups consisted of male patients who might be expected to show evidence of hormonal imbalance. In addition, a group of normal male control individuals was collected. From these various subjects, 24-hour urine specimens were assayed for a) chemically determined "estroids" and b) biologically determined estrogenic activity.

In another publication (Zarrow, Munson and Salter, 1950) will be found a collateral comparison of chemically determined 17-ketosteroids and biologically assayed androgens. Obviously, for multiple determinations such as these, relatively small samples only are available. In general, the equivalent of 6-hour excretions were employed.

Females

I. Early pregnancy. From patients making their first visit to the obstetrician, urine samples were obtained, and only those showing a positive Aschheim-Zondek test were used. One or two cases of threatened abortion also were included among this group of early pregnancies.

II. Ovulation. From a small group of normal women whose menstrual cycles had been followed carefully, the value for the probable time of ovulation was selected. In a later section of this communication will be found serial determinations for two such individuals at successive phases of the ovarian cycle.

Males

III. Control males. The male subjects comprised a rather wide range of ages, to correspond with the distribution among the male patients. Individuals were selected to avoid obvious endocrinopathies.

IV. Hypogonadism in males. From an ambulatory clinical and student population, individuals exhibiting delayed adolescence or adult eunuchoidism were selected. To the layman many of these individuals would appear normal. Most of the group, however, showed small or atrophic testes. oligospermia or aspermia, or poorly developed secondary sex characteristics. In a few cases the presenting clinical problem was chiefly psychiatric, complicated by a certain lack of male personality traits.

V. Hepatic disease. This group consisted of individuals with chronic liver disturbance. The patients tended toward subnormal testicular size and activity.

VI. Hepatic disease with gynecomastia. This group exhibited gynecomastia complicating chronic hepatic disease. Some decrease in testicular size and activity was the rule.

‡ These cases were studied by Dr. Anne Godley.

VII. Gynecomastia. From this group, individuals with fatty "pseudo-gynecomastia" were excluded. Each patient studied showed a firm button of fibrous edematous tissue underlying the areola (Salter, Klatskin and Humm, 1946; Karsner, 1946). Some individuals reported a trivial watery secretion from the tender nipple. Small testes and oligospermia were uniformly present, but no history of frank orchitis was obtained. Cases of prostatic cancer, or other individuals in whom gynecomastia was secondary to stilbestrol therapy, were excluded.

METHODS

Chemical determination of urinary "estroids."

Aliquots of urine amounting to at least one-eighth of the 24-hour specimen were subjected to acid hydrolysis, extraction with ether, and subsequent treatment to remove interfering pigments, according to the method outlined by Salter, Humm and Oesterling (1948). The clean, dried extracts were treated finally with Kober reagent (Venning *et al.*, 1937) for the development of the vermilion color; and shaken with organic solvents, as described in the same paper, in order to remove undesirable background pigments. The depth of color was read in the Beckman spectrophotometer at wave lengths 420, 520 and 620 millimicra in the visible spectrum, and the estrogen content calculated according to the formula given in the paper by Salter and Humm (in press). The "estroid" content was expressed as estrone-equivalents in micrograms.

Biologic assay of urinary estrogens.

The procedure for estimating estrogenic activity in the urine extracts was a modification of the method of Marrian and Parkes (1949) based on the vaginal response of spayed mice, and very similar to the procedure described by Mather (1942).

The phenolic fraction of the urine extract, in absolute alcohol solution, was reduced to a volume of 0.5 to 1.0 ml. (accurately measured) by evaporation under a stream of nitrogen, and to the alcoholic concentrate was added 9 volumes of distilled water with shaking. A clear solution or homogenous suspension suitable for injection resulted.

The total dose for each mouse was contained in 0.4 ml., divided into four injections of 0.1 ml. each, twice daily for two consecutive days. Total doses varying from 1/320 to 1/40 day's urine excretion could be prepared and administered readily in this manner. It may be noted that the injection volume was considerably smaller than that used by Mather (1942). The test mice were divided into groups of 5 or 6 individuals, and in each assay-series four groups were used for control injections of 10 per cent alcohol at three dosage levels of standard estrone solution (usually 0.035, 0.05 and 0.07 microgram). The treatments were rotated in each successive assay series so that the same group of mice did not receive the same treatment repeatedly. The mice were used for assay once every two weeks and in the intervening week received a "priming dose" of 0.10 microgram of estrone in a single injection.

Following the recommendations of Mather, vaginal smears were taken at 5 intervals following the fourth injection, and evaluated in the usual manner, wherein the absence of leukocytes and predominance of cornified cells indicated a positive effect. The response to the urine extract was estimated quantitatively by interpolation from the log-dosage response curve provided within every assay by the three respective groups of

mice which received three dosage levels of standard estrone. The urine extracts were assayed repeatedly, if necessary, until the response fell within the range of the standards.

By this procedure the three common estrogens showed the following potencies:

Estrone	1.0
α-Estradiol	1.75
Estriol	0.5

EXPERIMENTAL FINDINGS

A synopsis of the experimental observations is presented in three charts. In Figs. 1 and 2, respectively, the chemical "estroid" values and the estro-

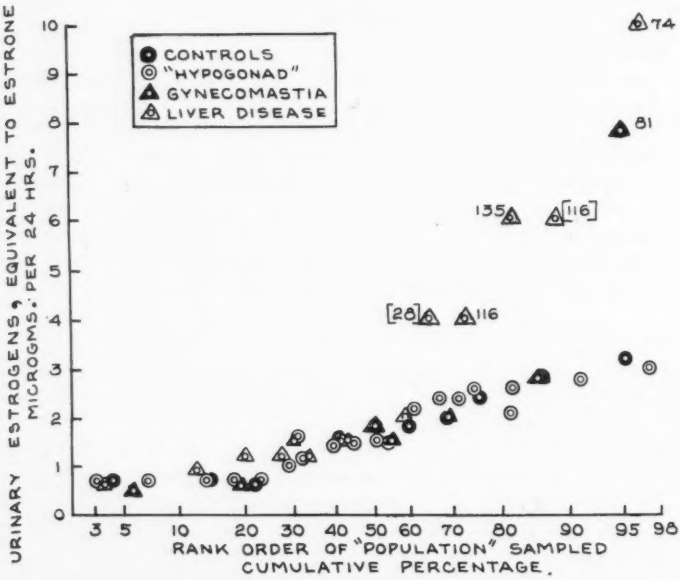

FIG. 1. Arrays of several groups of individuals, showing daily excretion of urinary estrogens as determined by biologic assay. The normal control males and "hypogonad" males merge. Distinct from these (in the upper limb of the population) are the cases of liver disease and gynecomastia (males). Even more distinct are ovulating females, but these fall above the top of the graph. In each array the data are arranged in order of increasing magnitude.

gen assays for each group are plotted in "rank-distribution." This presentation shows not only the differences between the means of each group, but also surveys the range of the individual values. The "rank-distribution" is a well-established biometric procedure (Fisher and Yates, 1943), which merely arranges a series of measurements in order of increasing size. The respective data are spread out on a percentile scale, and the position

of each figure on this scale depends upon the total number of cases in the group. The scale is so adjusted that the mean value falls near the 50-percentile mark.

In Fig. 1 the bioassays for estrogens are shown. It will be noted that the values for normal controls and for the hypogonadal patients form merging populations, as confirmed by appropriate statistical calculations. At the higher levels of excretion, the hepatic and gynecomastia values rise de-

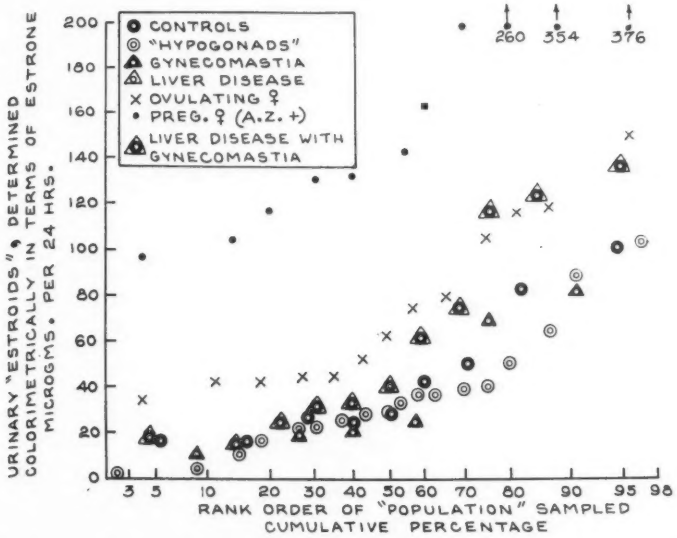

FIG. 2. Arrays of several groups of individuals, showing daily excretion of urinary "estroids" as determined microchemically. The normal control males and "hypogonad" males merge. Distinct from these, especially in the upper limb of the population, are cases of liver disease and gynecomastia (males). Even more distinct are the ovulating females and women in early pregnancy. In each array the data are arranged in order of increasing magnitude.

cisively above those of the control group. Still higher lies the level reported by Smith and Smith, (1935) for ovulating females, but this range is off scale as confirmed by a few iolated determinations of our own.

The data for chemically determined "estroids" are presented in Fig. 2. As in Fig. 1, the values for normal males and hypogonad males merge into one population. The hepatic and gynecomastic cases, however, rise distinctly above the normal group,—especially in the upper limb of the population. In this respect the chemical values reflect the bioassays. Moreover, the chemical values for ovulating females and for cases of early

pregnancy (lying at higher levels) also show a tendency toward an exaggerated up-swing of the higher limb of the population.

CORRELATION BETWEEN BIOLOGICALLY DETERMINED ESTROGENS AND CHEMICAL VALUES FOR "ESTROIDS"

In Fig. 3 the chemical and biologic observations are charted for the several groups. Estrogens (assayed biologically) are plotted against the ordinate, and "estroids" (determined chemically) against the abscissa, both in terms of equivalent amount of estrone. The computed correlation

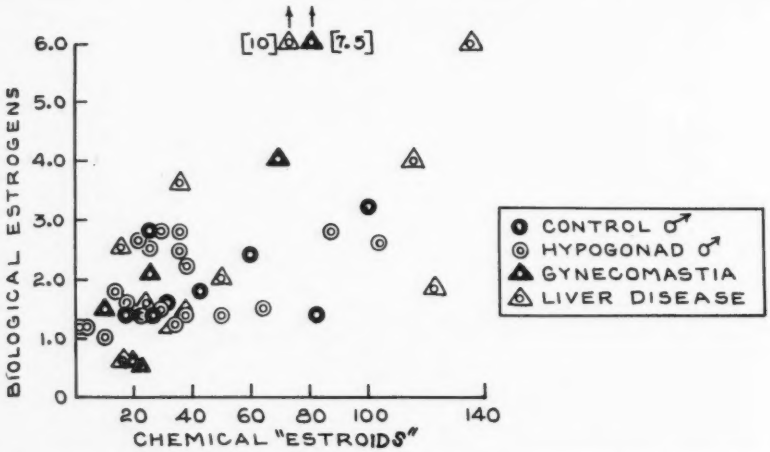

FIG. 3. Correlation between biologic assays of urinary estrogens and microchemical determinations of "estroids." Note that the combined populations of normal and "hypogonad" males show an extremely low potency-equivalent per chemical unit, i.e., 0.01. The over-all correlation coefficient, $r = 0.52$.

coefficient for all data is 0.52, i.e., of borderline significance. When individual groups from this mixed population are selected, however, better correlation is found. For example, when groups V and VII are combined, the correlation coefficient becomes 0.76. Moreover, it can be shown statistically that this combined group is quite distinct from ovulating or pregnant females (groups I and II) or from the males of groups III and IV. Among the groups last named, the correlation is poor ($r = 0.45$); but the values involved are so close to the baseline that one could hardly expect a significant trend at such low levels.

Of special interest is the upswing in the higher levels of these populations. This departure from the normal distribution is consistent with the finding of Engel *et al.* (1950) that purified urinary extracts contain more than one

photogenic component. Indeed, these colorimetric data confirm the presence in males of low potency material, corresponding to that pronounced by Engel *et al.* (1950) as "non-specific." In this respect the data obtained in different laboratories by different methods agree.

As shown in Table 1, by calculating the regression coefficient for the several groups plotted in Fig. 3, it is possible to determine the over-all endocrine potency per Kober color unit for each group. When this is done, it is seen that for normal males a Kober-color unit (expressed as estrone) implies very little biological activity; whereas in nutritional gynecomastia

TABLE 1. BIOLOGICAL POTENCY PER CHEMICAL COLOR UNIT FOR URINARY ANALYSES, WITH CORRELATION OF BIOLOGICAL AND CHEMICAL VALUES

Subjects			Potency		Correlation	Grand synopsis	
						Potency	Correlation
Group	Status	No. Cases	b	b	r	b	r
FEMALES							
I	Early Preg.		1+(estimated)				
II	Ovulation		1+(estimated)				
MALES							
III	Control	9	0.010	0.010	0.45		
IV	"Hypogonad"	18	0.010				
V	Hepatitis	7				.028	0.52
VI	Hepatitis and gynecomastia	4	0.031	0.037	0.57		
VII	Gynecomastia	6	0.085				
	Total	44					

r = Coefficient of Correlation.
b = Potency Conversion Factor.

the potency per color unit is definitely higher. The same is even more obvious for ovulating females.

Further substantiation of the chemical procedure is afforded by isolated values obtained in abnormal circumstances, in females. These are shown in Table 2. It will be noted that in the case of early abortion, the chemical value rose and fell. In the amenorrhea of hypocorticism (Addison's disease), a low value was encountered, and this was also true of pituitary infantilism. On the other hand, a high chemical value was encountered in juvenile adrenal virilism, but this "estroid" showed very little biological activity. Obviously, conversion factors must be selected for individual syndromes in the light of qualitative studies of the respective steroid mixtures excreted. For any well-defined group, e.g., normal females, it appears justifiable to assume an appropriate conversion factor. Under such circumstances, as illustrated by the cycles of two women shown in Figs. 4 and 5,

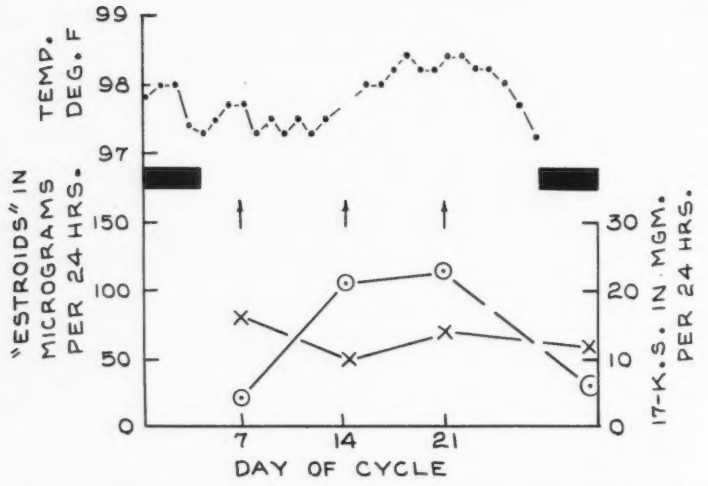

FIG. 4. Chemical analyses of urinary 17-ketosteroids (designated by ×) and of "estroids" (designated by dot in circle) during a single ovarian cycle of a normal woman aged 25. The black bars indicate the duration of menstruation.

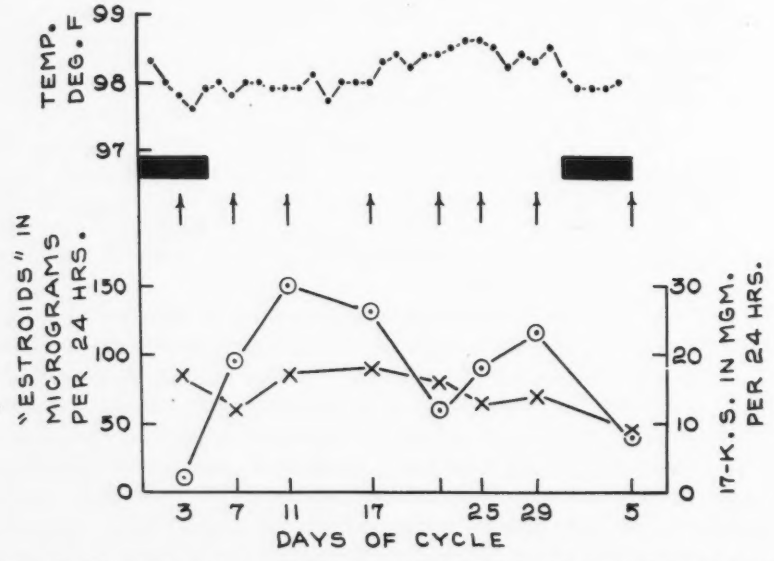

FIG. 5. Chemical analyses of urinary 17-ketosteroids (designated by *x*) and of "estroids" (designated by dot in circle) during a single ovarian cycle of a normal woman aged 22. The black bars indicate the duration of menustration. Of special interest is the secondary rise in "estroids" during the luteal phase.

the chemical procedure probably gives as much information as the longer and more laborious bioassay of the mixed urinary metabolites.

DISCUSSION

For many purposes of metabolic and clinical investigation the precise qualitative identification of individual estrin catabolites from large volumes of urine is not essential. What is required in many physiological

TABLE 2. CHEMICAL ANALYSES OF HUMAN URINARY STEROIDS IN SPECIAL CLINICAL CASES (FEMALES)

Patient	Diagnosis	17-Ketosteroids mg./24 hr.	"Estroids" μg. ⇔estrone/24 hr.
Ce. 34 preg. 6 wks.		15.9 8.0	20 May 22 30 May 27
Rt. 31 preg. 4 mos.	Threatened Interrupt. Preg.	11.1	1040
Mi. preg. 3 mos.	Threatened Abortion	5.3	77
Pa. preg.	Threatened Abortion (did not abort)	3.6 4.4 4.8	109 March 25 146 March 28 151 April 7
Ka. 21 infantile	Infantilism	1.4	12
Br. 6	Juvenile Adrenal Virilism	33.4	95
Gr. 9	Precocious Puberty	1.1	12.8
Cs. 42	Addison's Disease	0.6	10
Gh. 28 preg. 3 mos.	Bleeding Abortion Post Op. D & C	14.9 9.9 9.3	27 April 20 36 May 5 21.6 May 27

studies of the female cycle is the analysis of estrogen metabolism over a rather short time interval. Furthermore, the total amount of labor involved must be minimal if large numbers of determinations are to be made. Since the publication of Venning *et al.* in 1937, several procedures have been advanced which employ the Kober reagent (Salter *et al.* 1948; Stimmd, 1946; Bachman and Pettit, 1941). Hitherto this colorimetric determination has been plagued by the presence of undesirable background pigments (Oseterling and Salter, 1947). For this reason fluorometric methods (Jailer, 1947; Bates and Cohen, 1947; Finkelstein, Hestrin and Koch, 1947; Finkelstein, 1948) were developed in the hope of increasing the

specificity of the final photo-electric effect. Ironically, with either terminal procedure another difficulty presents itself in male urine, namely, the presence of a chromogen of low biological potency.

This chromogen, described by Engel *et al.* (1950) as "non-specific," is shown by the Kober technique to differ from the classical "undesirable background pigment." In addition, the biological assays reported herewith raise the possibility that this chromogen (although nearly inert) may have an endocrinological significance. One must give thought to a correlation coefficient of 0.52 among the groups of males studied, and to higher values obtained both for biological potency and for chemical titre in the cases of hepatitis and gynecomastia. It is clear, however, that even in such cases, the factor for biological color equivalence in males is at least twenty times lower than for normal females. Therefore, equal color titres in male and female urine must be interpreted differently in the light of the qualitative disparity of the steroid chromogens in the respective urines. This is true both for the present report and for the fluorometric determinations by Engel *et al.* (1950) following partition by the method of counter-current distribution. A similar but less drastic qualitative disparity was encountered by Zarrow *et al.* (1950) in studying the 17-ketosteroid excretion of gynecomastic patients as compared with normal males.

If the qualitative nature of the urinary steroid catabolites can be inferred from the type of individual being studied, the Kober procedure reported herewith can be applied to samples of urine representing only four to six hours of excretion. This fact is demonstrated by the curves for ovarian cycles and the data on abortion included herewith. Obviously in repeated and protracted physiological investigations, a precise chemical identification of each of the possible steroid catabolites is no more feasible than the separation of the individual nitrogenous constituents of urine in nutritional studies.

Of course, qualitative identification of the major steroid components in well recognized physiological states and clinical syndromes will be desirable (Gallagher, 1951). Characteristic values of androgenic potency will also be helpful. This point is illustrated by the studies of Zarrow *et al.* (1950) on androgens in gynecomastia and liver disease. Once this qualitative description has been made, however, the further use of composite chemical categories would appear justified; because complex separations and biological assays can add little further information.

In the meantime it will be important to identify the low-potency material in male urines disclosed by the Kober procedure in the present studies, because it appears identical with the "non-specific" material of Engel *et al.* (1950) and possibly with the interfering pigment of Stevenson and

Marrian (1947). It appears to be quite distinct from the classical brownish melanin-like interfering substance.

With respect to the excretion of hormones in malnutrition, Valero, Reyes and Mont (1950) have reported a temporary rise in gonadotrophin response to improved nutrition, and with this a transitory outburst of of estrogen excretion.

SUMMARY

The correspondence of biologic assays of estrogens and microchemical determinations of "estroids" by a modified Kober procedure has been examined for human urine obtained from several groups of patients and normal individuals. In general, these studies cover the so-called "physiologic" range of estrogen excretion. The female subjects included amenorrheic, ovulating, and early pregnant individuals. The male subjects comprised five groups, i.e., normal controls, hypogonadism, hepatic disease, hepatic disease with gynecomastia, and nutritional gynecomastia. Over this low physiological range for males the correlation ($r=0.52$) was only suggestive. The normal male population, however, showed very low color values with almost no biologic potency. Cases falling above this range showed the same correlation ($r=0.57$) with a higher biologic potency.

Because the ratio of estrogens (biologic) to "estroids" (chemical) is higher in ovulating females and in gynecomastic males than in normal males, a qualitative difference in the urinary steroid metabolites is indicated. When definite categories of individuals are selected, the microchemical determination of "estroids" reflects adequately the biologic assay of urinary estrogens.

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NOTES AND COMMENTS

OUABAIN AND THE ADRENAL CORTEX[1]

The cardiac glycoside, ouabain, and the steroid desoxycorticosterone acetate are related structurally. The two drugs have opposing effects on the electrocardiogram (Kupperman and DeGraff, 1950). Moreover, whereas desoxycorticosterone alone induces potassium depletion from cardiac muscle in rats, the concomitant administration of ouabain and desoxycorticosterone has no such effect (Gardner, Berman and Butler, in preparation). The adrenal glands from the rats studied in the experiments of Gardner *et al.* were preserved for histological and histochemical study. Desoxycorticosterone has been found by several different investigators to induce atrophy and lipid depletion of the outer portion of the cortex, the zona glomerulosa (for refs., see Greep and Deane, 1947). The appearance of this zone was therefore studied in frozen sections of formalin-fixed glands stained for lipids with sudan black B.

Thirty-six adult male rats were divided into 6 groups. The control animals received no injections. The rest received daily subcutaneous injections of desoxycorticosterone acetate (DCA)[2] in aqueous suspension, ouabain (DIG)[2] in aqueous solution, or both together for 30 days, as shown in the accompanying table. The average width of the zona glomerulosa is given as well, and its histochemical appearance in the various specimens of each group is summarized. The glomerulosa was measured on outlines drawn with the aid of a camera lucida at 750×.

TABLE 1. INFLUENCE OF DESOXYCORTICOSTERONE ACETATE (DCA) AND OUABAIN (DIG) ON THE WIDTH AND LIPID CONTENT OF THE RAT'S GLOMERULOSA

Injection	Width of glomerulosa, μ	Lipid content of glomerulosa
none	46	moderate
DCA	29	none
DIG-a	59	high
DIG-a + DCA	52	low
DIG-b	55	high
DIG-b + DCA	58	low

DCA: 5 mg/day.
DIG-a: 3.4 mg/kilo/day.
DIG-b: 6.0 mg/kilo/day.

Desoxycorticosterone alone induced both atrophy and depletion of the glomerulosa. Ouabain alone produced enlargement of this zone as well as a conspicuous increase in its lipid content. When the two drugs were administered together, the atrophy caused by

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[1] This investigation was supported in part by a Cooperative Research Grant from the National Heart Institute, National Institutes of Health, U. S. Public Health Service, Federal Security Agency.

[2] Desoxycorticosterone acetate (Percorten) was generously made available by Dr. Fred E. Houghton, Research Division, Ciba Pharmaceutical Products, Summit, N. J., and Ouabain (Strophanthin-G) by S. B. Penick and Co., New York, N. Y.

DCA was offset, although the lipid content of the glomerulosal cells was lower than normal in most of the specimens.

No final explanation for this apparent antagonism between these two drugs on the adrenal cortex can be reached without further experimentation. Desoxycorticosterone, although possibly not a normal hormone of the adrenal cortex, induces sodium retention and potassium excretion. It thus simulates the activity of certain extracts of the adrenal cortex. Greep and Deane postulated that the atrophy of the glomerulosa following administration of this steroid might reflect disuse because of its substitution for the normal products of the zone. They suggested that the disuse atrophy might depend on the electrolyte shift induced by desoxycorticosterone. Supporting evidence for that hypothesis has come from Deane, Shaw and Greep (1948), who showed that a similar atrophy follows potassium depletion, and from subsequent workers, who have found it with sodium "flooding" (Badinez and Croxatto 1948; Knowlton et al. 1949; Bacchus 1950). One possible explanation for the present observation would therefore be that ouabain may displace the electrolyte balance in a fashion opposite to desoxycorticosterone. Gardner *et al.*, however, found that when ouabain is given alone it does not increase cardiac muscle potassium. This observation indicates that the general action suggested by Greep and Deane (1947) is improbable, although there are conflicting data in the literature as to whether therapeutic doses of the digitalis glycosides do or do not cause an increase in cardiac muscle potassium (cf. Wedd 1939; Boyer and Poindexter 1940).

Another, perhaps more attractive, hypothesis derives from the fact that the aglucone of ouabain and desoxycorticosterone are structurally similar. The two steroids may thus compete for local enzyme systems. In cells of the glomerulosa as well as in cardiac muscle fibers, desoxycorticosterone may enter a molecular arrangement in performing its local activity. If that position were occupied by a structural analogue with different metabolic abilities, the local effect of desoxycorticosterone would be prevented.

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ASSOCIATION NOTICES

To the Editor of the *Journal of Endocrinology*

Dear Sir:

Through you I should like to discuss with your readers, and particularly with the members of the Association for the Study of Internal Secretions, the type of program which we are planning for Atlantic City in June of 1951.

At our meeting in San Francisco we voted to have three days of scientific programs, rather than the two we have had in the past. Dr. Robert Williams, of Seattle, Washington, wrote and suggested that we try grouping the papers under separate headings, and this suggestion met with favor in the Council meeting.

Accordingly, we have made arrangements for Thursday, Friday and Saturday, June 7th, 8th, and 9th, the meetings to be held either in the Chalfonte or Haddon Hall hotels. Tentatively, we are suggesting the following schedule:

THURSDAY: 9:30 A.M.—General subjects.
2:00 P.M.—Papers relating to the pituitary body.
8:00 P.M.—"Information Please," a program of informal discussion with the audience and a "Board of Experts" taking part.
FRIDAY: 9:00 A.M.—Papers relating to the thyroid and parathyroid glands.
2:00 P.M.—Papers relating to the adrenal glands.
Evening —The annual cocktail party and banquet.
SATURDAY: 9:00 A.M.—Papers relating to gonads and sterility.
2:00 P.M.—Joint session with the American Diabetes Association.

For each of these sessions there will be two presiding officers; one interested principally in clinical medicine, the other in experimental studies. These doctors will be responsible for the selection of papers for their own program, the title and abstracts being sent to them by the chairman of the Program Committee.

This letter is accompanied by instructions for the submission of abstracts. These instructions were prepared by Dr. Edward Doisy. Last year he and his associates and their secretaries had several weeks of needless work due to the incompleteness of information. Dr. Jane Russell, School of Medicine, Emory University, Georgia, is chairman of the Program Committee for 1951. We are particularly desirous of having more papers relating to clinical medicine submitted for these programs. The early submission of titles and complete abstracts will greatly facilitate planning of our meeting. Because so many members of the Program Com-

mittee will need to review these abstracts, we must continue to ask for four copies. No abstracts received after March 1, 1951, can be considered.

We are going to try this three-day meeting for 1951. The course to be followed in subsequent years will be determined by vote at the annual meeting at the conclusion of this program.

THE 1951 AWARDS OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

FELLOWSHIPS

The Ayerst, McKenna and Harrison Fellowship

The Ayerst, McKenna and Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin and in 1948 to Dr. Ernest M. Brown, Jr. Dr. Brown was re-elected for the Fellowship in 1949, and the 1950 Fellowship was awarded to Dr. Lawrence E. Shulman.

The Schering Fellowship in Endocrinology

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson, who also received the 1950 Fellowship.

Association Fellowships are designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $2,500.00, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

AWARDS

The E. R. Squibb and Sons Award

The E. R. Squibb & Sons Award of $1,000.00 was established in 1929, and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; and in 1950 to Dr. C. N. H. Long. In 1943 no award was given. A special committee of five members of the Association selects

the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; and in 1950 to Dr. Oscar M. Hechter. The Award is for $1,200.00. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $1,800.00.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City 3, Oklahoma, and returned to him not later than March 15, 1951.

THE 1951 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-third Annual Meeting of the Association for the Study of Internal Secretions will be held at the Chalfonte-Haddon Hall Hotel, Atlantic City, New Jersey. The Scientific Sessions have been extended to three days, Thursday, Friday, and Saturday, June 7-8-9.

The Local Chairman of Arrangements is Dr. Matthew Molitch.

Due to the advance in the date of our meeting, Chalfonte-Haddon Hall Hotel will be unable to accommodate all of our members by reason of a previous commitment. The Colton Manor, on Pennsylvania Avenue near Haddon Hall, is an excellent hotel, which has been recently refurnished, and is holding one hundred rooms for our members on the "first come, first served" basis. The Hotel Strand, at Pennsylvania Avenue and the Boardwalk, has also promised to supply a limited number of rooms. It is important that reservations be made immediately if you are to avoid disappointment.

All Scientific Sessions will be held in the Chalfonte-Haddon Hall Hotel. The rooms, in which the session will be held, will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, June 8, at 7:30 P.M. in the Rutland Room, preceded by cocktails at 6:30 P.M. in the adjoining Benjamin West Room.

Those wishing to present papers, which will be limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Jane Russell, School of Medicine, Emory University, Atlanta, Georgia, not later than March 1, 1951. *It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing on the program and journals of the Association.* The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program.

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, i.e., M.D., of each author should be given after his name.
   Line 3. Institution of origin; city in which institution is located.

3. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.

Nominations for the Ayerst, McKenna and Harrison Fellowship, the Schering Fellowship in Endocrinology, and the Squibb and Ciba Awards should be made on special application forms, which may be obtained from the Secretary-Treasurer, Henry H. Turner, M.D., 1200 North Walker Street, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1951.

EDWARD H. RYNEARSON, *President*